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Cyclin E1 overexpression triggers interferon signaling and is associated with antitumor immunity in breast cancer

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ABSTRACT

Background Cyclin E1 overexpression drives oncogenesis in several cancers through deregulation of DNA replication and induction of genomic instability, which may potentially trigger immune signaling via cytoplasmic DNA. However, the effects of cyclin E1 overexpression on tumor immunity and its effects on the response to immune checkpoint inhibitors remain largely unclear.

Methods Tissue microarrays and clinical outcomes of 398 patients with breast cancer were analyzed to explore the correlation between cyclin E1 expression, patient survival, and immune cell infiltration using immunohistochemistry. Genomic data from publicly available data sets and three clinical trials evaluating immunotherapy were assessed to measure the impact of cyclin E1 expression on the immune cells in the tumor microenvironment and response to immunotherapy in patients with breast cancer. In addition, breast cancer cell lines with inducible cyclin E1 overexpression were employed to analyze the effects of cyclin E1 on inflammatory signaling.

Results Increased cyclin E1 expression in breast cancer was positively correlated with immune cell infiltration, including T cells, B cells, and natural killer cells, and activation of interferon-related pathways. Importantly, higher cyclin E1 expression or *CCNE1* amplification was associated with better response to immunotherapy in three clinical trials. Mechanistically, cyclin E1 overexpression resulted in micronuclei formation and activation of innate immune signaling, resulting in increased immune cell migration.

Conclusions Our data show that cyclin E1 overexpression associate with antitumor immunity through activation of innate inflammatory signaling and warrants investigation into amplification or overexpression of cyclin E1 in identifying patients with breast cancer eligible for immunotherapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ *CCNE1* amplification, as one of the major sources of replication stress in cancer cells, is known to induce genomic instability. Genomic instability can trigger immune signaling via cytoplasmic DNA. However, whether cyclin E1 overexpression triggers inflammatory signaling and has effects on tumor immunity and response to immune checkpoint inhibitors in patients with breast cancer remains largely unclear.

WHAT THIS STUDY ADDS

⇒ Increased cyclin E1 expression in breast cancer positively correlates with immune cell infiltration and better response to immunotherapy.

⇒ Cyclin E1 overexpression results in micronuclei formation and activation of innate immunity, resulting in increased immune cell migration in breast cancer.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Immunotherapy might offer opportunities for therapeutic interventions in breast cancer patients with cyclin E1 overexpression or amplification.

BACKGROUND

One in eight cancer cases diagnosed globally concerns breast cancer, making it one of the most frequently diagnosed cancer types.[1] Over the recent years, immune checkpoint inhibitors (ICIs), including anti-programmed death 1 (PD-1), anti-programmed death ligand 1 (PD-L1), and anti-cytotoxic T-lymphocyte associated protein 4, have demonstrated significant therapeutic effects in various cancer types, including melanoma and non-small cell lung cancer.[2 3] Although its incorporation into breast cancer treatment has been more gradual, the combination of pembrolizumab with chemotherapy showed efficacy in a subset of patients with triple-negative breast cancer (TNBC) whose tumors expressed PD-L1 with a combined positive score (CPS)≥10.[4] The observed response in only a subset of patients might be related to the heterogeneous tumor immune microenvironment across and within breast cancer

subtypes, although it remains unclear which patient or tumor features determine a response. However, given that promising results were observed in a subgroup of patients with breast cancer, identifying the molecular characteristics that led to response to ICI treatment in patients with breast cancer is imperative.

Cyclin E1 (encoded by *CCNE1*) is a proto-oncogene that controls the transition from the G1 to S phase of the cell cycle. Specifically, cyclin E1 functions as the cognate cyclin partner of cyclin-dependent kinase-2 (*CDK2*), which drives the onset of DNA replication.[5] Dysregulation of the cyclin E1/Cdk2 complex via cyclin E1 overexpression provokes premature entry into the S phase through increased firing of replication origins, leading to conflicts between the replication and transcription machineries, thereby resulting in DNA damage,[6 7] subsequent mitotic aberrancies,[8 9] and genomic instability. In line with these findings, amplification or overexpression of cyclin E1 has significant clinical implications, with *CCNE1* amplification being associated with poor prognosis in patients with breast cancer.[10 11]

Genomic instability can lead to chromosome mis-segregation during mitosis and a subsequent interferon response through micronuclei formation.[12 13] The cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway acts as a key mediator between DNA damage and innate immunity. Specifically, the DNA sensor cGAS directly detects cytosolic double-stranded DNA and activates STING, thereby inducing a type I interferon (IFN) response through the phosphorylation of the IRF3 transcription factor.[14 15] Through these mechanisms, genomic or chromosomal instability can trigger cGAS-STING-mediated innate immunity and recruitment of CD8+T, and natural killer (NK) cells.[16 17] Because of its pro-inflammatory capabilities, the cGAS-STING pathway also leads to upregulation of PD-L1 expression.[18 19] Based on these findings, therapeutic modulation of the cGAS-STING pathway has previously been linked to response to ICIs, especially in BRCA-deficient breast cancer.[20 21]

Whether cyclin E1 overexpression, which leads to genomic instability, is also associated with features of the tumor microenvironment (TME) and immune activation is largely unclear. In this study, we aimed to investigate whether cyclin E1 amplification or overexpression correlates with antitumor immunity in breast cancer.

METHODS

Patient cohorts and tissue samples

A retrospective cohort of 398 patients with primary breast cancer from the University Medical Center Groningen was analyzed using tissue microarrays (TMAs) to investigate the relationship between cyclin E1 and the tumor immune microenvironment. Immunohistochemical (IHC) staining assessed cyclin E1, CD4, CD20, CD57, PD-L1, and CXCL11 expression in our cohort. The details of patient selection and IHC staining are provided in Online supplemental methods.

For The Cancer Genome Atlas (TCGA), Memorial Sloan Kettering (MSK) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) breast cancer cohorts, data were downloaded through cBioPortal (https://www.cbioportal.org/). For the I-SPY2 trial, data were downloaded through GSE173839 and GSE194040 in Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo). For the multicentral IMvigor210 trial, data were downloaded from IMvigor210 (http://research-pub.gene.com/IMvigor210CoreBiologies). Data processing and bioinformatics analysis details for each cohort are provided in the Online supplemental methods.

Cell line-based experiments

A detailed description of each experiment is provided in the Online supplemental methods. In brief: HCC1806 and HCC38 cell lines were cultured to stably overexpress or knock down CCNE1. Protein expression levels of CCNE1, pSTAT1, STAT1, and pIRF3 were analyzed using western blotting, while immunofluorescence microscopy was employed to visualize cGAS localization in cells. Gene expression levels of immune-related markers (CXCL9, CXCL10, CXCL11, IFN-γ, and IFNB1) were quantified using quantitative reverse transcription PCR. Peripheral blood mononuclear cells (PBMC) migration assays were conducted using transwell assay, with CXCL11 blockade and flow cytometry to characterize immune subsets. Additionally, flow cytometry was also used to evaluate PD-L1 expression in HCC1806 cells.

Statistical analysis

All statistical analyses were conducted using R V.4.0.4 and Prism (V.9) software (GraphPad). Results were considered statistically significant when $p<0.05$. Detailed descriptions of the statistical methods are provided in the Online supplemental methods.

RESULTS

Cyclin E1 correlated with poor clinical outcomes in patients with breast cancer

To explore the clinical relevance of cyclin E1 in patients with breast cancer, IHC staining for cyclin E1 was performed on TMAs (figure 1A). The expression levels of cyclin E1 were scored separately for cytoplasmic and nuclear staining (figure 1B). Survival analysis revealed that patients displaying combined high nuclear and high cytoplasmic cyclin E1 expression or either high nuclear or high cytoplasmic expression were significantly associated with poor relapse-free survival (RFS) in our patient cohort (figure 1C–E). Furthermore, multivariate Cox regression analysis showed that combined high cytoplasmic and high nuclear cyclin E1 expression was independently associated with poor RFS after adjusting for molecular subtype, tumor infiltrating lymphocyte (TIL) levels, and clinical stage, something that was not seen for cases that displayed either high nuclear or high cytoplasmic cyclin

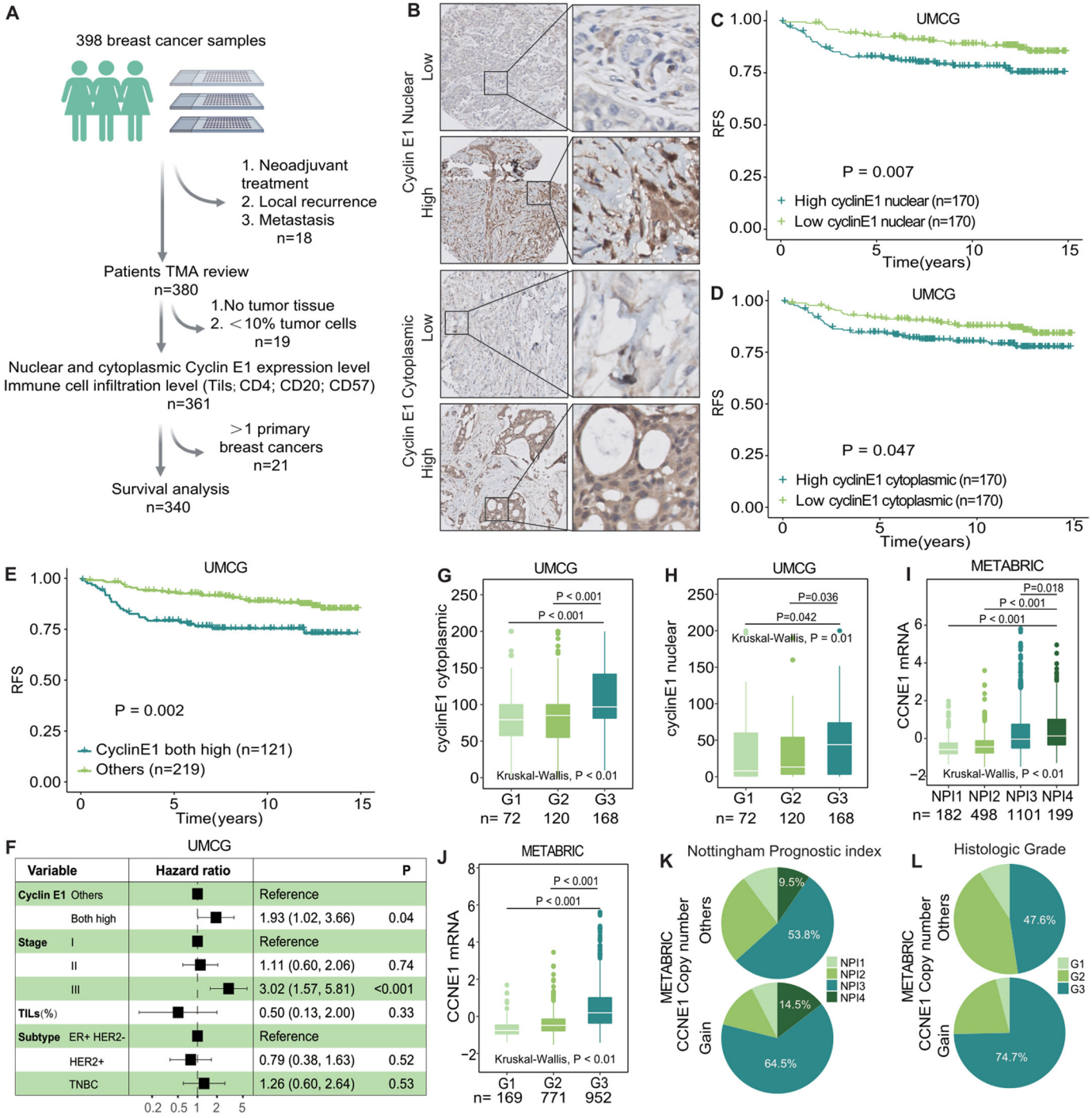

Figure 1 Cyclin E1 correlated with poor clinical outcome in patients with breast cancer. (A) Flow diagram of sample selection (created with BioRender.com). (B) Representative images of cyclin E1 (nuclear) and cyclin E1 (cyto) expression in patients with breast cancer's TMAs. (C–D) Kaplan-Meier survival analysis of patients with high and low levels of cyclin E1 nuclear (RFS; log-rank test) or cytoplasm (RFS; log-rank test) expression in our TMAs. (E) Kaplan-Meier survival analysis of patients with cyclin E1 cytoplasm and nuclear both high (RFS; log-rank test) expression in our TMAs. (F) Multivariable Cox regression analysis of RFS in our patient TMAs cohort. Data are presented as HR and 95% CI. (G–H) The comparison of cyclin E1 cytoplasm (G) and nuclear (H) expression in different tumor grades. Statistic test: Kruskal-Wallis test with the Dunn's multiple-comparison test was used for comparisons among groups. (I–J) The comparison of cyclin E1 mRNA expression in different NPI and tumor grades in the METABRIC database. Statistic test: Kruskal-Wallis test with the Dunn's multiple-comparison test was used for comparisons among groups. (K–L) The percentage of different NPI and tumor grades between patients without or with CCNE1 copy number gain in the METABRIC database. METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; mRNA, messenger RNA; NPI, Nottingham Prognostic Index; OS, overall survival; RFS, relapse-free survival; TIL, tumor infiltrating lymphocyte; TMAs, tissue microarrays; TNBC, triple negative breast cancer; UMCG, University Medical Center Groningen.

E expression (figure 1F, online supplemental figure S1A,B). Of note, survival analysis also revealed that high cyclin E1 messenger RNA (mRNA) expression was significantly associated with poor overall survival (OS) both in the TCGA and METABRIC cohorts (online supplemental figure S1C,D). Since *CCNE1* amplification is an oncogenic driver,[22 23] *CCNE1* copy number alterations (CNAs) were explored in public cohorts. We first explored the distribution of *CCNE1* copy number events in the TCGA pan-cancer cohort and found that *CCNE1* copy number amplification was most common in gynecologic cancers and gastric adenocarcinomas (online supplemental figure S1E). We then checked whether *CCNE1* copy numbers were associated with cyclin E1 mRNA expression and noticed that compared with *CCNE1* deletion or *CCNE1* "neutral" tumors, tumors with a *CCNE1* copy number gain or amplification showed higher expression of cyclin E1 mRNA in both the TCGA and METABRIC cohort (online supplemental figure S2A,B). In addition, *CCNE1* amplification was significantly associated with worse OS in the MSK cohort and worse recurrence-free survival in the METABRIC cohort (online supplemental figure S2C,D). However, in the METABRIC and TCGA breast cancer cohort, worse OS was not observed in the *CCNE1* amplification group (online supplemental figure S2E,F). To explore the prognostic value of *CCNE1* amplification across cancer types, we analyzed two tumor types in the TCGA pan-cancer cohorts in which *CCNE1* is commonly amplified. Consistent with our observations in the patient with breast cancer data, patients with ovarian cancer harboring a tumor with *CCNE1* amplification showed shorter survival, whereas patients with gastric adenocarcinoma harboring *CCNE1* amplification did not (online supplemental figure S2G,H).

To further explore the clinical implications of cyclin E1 expression in patients with breast cancer, we analyzed its correlation with tumor grade and proliferation. Higher cytoplasmic and nuclear cyclin E1 levels were more frequently found in the tumors with G3 histological grade (figure 1G,H). Additionally, positive associations between both cytoplasmic and nuclear cyclin E1 and Ki-67 proliferation index were observed (online supplemental figure S3A,B). Furthermore, in the METABRIC cohort, the mRNA levels of cyclin E1 were increased in higher Nottingham Prognostic Index (NPI) grade and tumor grade groups (figure 1I,J). In line with these data, breast cancers with *CCNE1* copy number gain showed a higher percentage of high tumor NPI grade and high histologic grade (figure 1K,L). Importantly, we noticed that cyclin E1 mRNA expression was weakly negatively associated with age in patients with breast cancer in both TCGA and METABRIC databases (online supplemental figure S3C,D). Combined, these findings consistently showed that elevated cyclin E1 expression was associated with unfavorable tumor characteristics and worse prognosis.

Cyclin E1 expression is correlated with immune cell infiltration in the tumor microenvironment in patients with breast cancer

To investigate the effects of cyclin E1 expression on immune features in the microenvironment of breast cancers, we first analyzed TIL patterns within the TCGA database. TIL patterns were classified into one of five categories, according to a recent pan-cancer spatial organization analysis[24] (online supplemental figure S4A). Notably, tumors with high cyclin E1 mRNA expression correlated with two "brisk" patterns, and the *CCNE1* copy number gain group also showed a trend towards an increased proportion of "brisk band-like" patterns of TILs (figure 2A,B). Tumors in the TCGA cohort with *CCNE1* copy number gain consistently demonstrated significantly higher levels of TILs percentage (figure 2C). Importantly, IHC analysis of our patient cohort also revealed a positive association between cyclin E1 cytoplasmic expression and TIL numbers in all, human epidermal growth factor receptor-2 positive and estrogen receptor-positive and HER-2 negative cancers, but not in TNBCs (figure 2D,E). This discrepancy may be attributed to the relatively low number of patients with TNBCs in the cohort. Of note, compared with tumors with both low cytoplasmic and low nuclear cyclin E1 expression, cytoplasmic cyclin E1 high expression groups exhibited higher levels of TILs (online supplemental figure S4B).

To delineate the role of *CCNE1* copy number gain in shaping an antitumor immune phenotype, we compared gain of *CCNE1* or other replication stress-related oncogenes in relation to the expression of a panel of immunomodulatory genes in the TCGA cohort. Among these proto-oncogenes, the *CCNE1* gain group showed increased expression of immunomodulatory genes, including ICOS and IFN-stimulated genes CXCL9, CXCL10 and CCL5 (figure 2F). Of these genes, ICOS plays a critical role in the development of follicular helper T cells, while CXCL9 is involved in T-cell recruitment, and CXCL10 and CCL5 are chemokines associated with the type I IFN response.[25 26] Since immune cell receptor expression reflects an immune cell-activated TME in multiple cancers,[27] we next explored the relation between oncogene status and the richness and diversity score of the T-cell receptor or B-cell receptor. The richness and diversity scores of immune cell receptor repertoires were increased in the *CCNE1* gain group when compared with cancers lacking *CCNE1* gain (figure 2G). This increase was also observed with other cancers with mutation or CNA of oncogenes, but the difference was more significant in tumors with a *CCNE1* CNA change (online supplemental figure S4C).

To further elucidate the immune cell composition within the microenvironment of breast cancers with a *CCNE1* gain, we performed deconvolution analysis of the bulk RNA sequencing (RNA-seq) data from both TCGA and METABRIC cohort, using Microenvironment Cell Populations-count (MCP-counter) and single sample gene set enrichment analysis (ssGSEA) analysis.

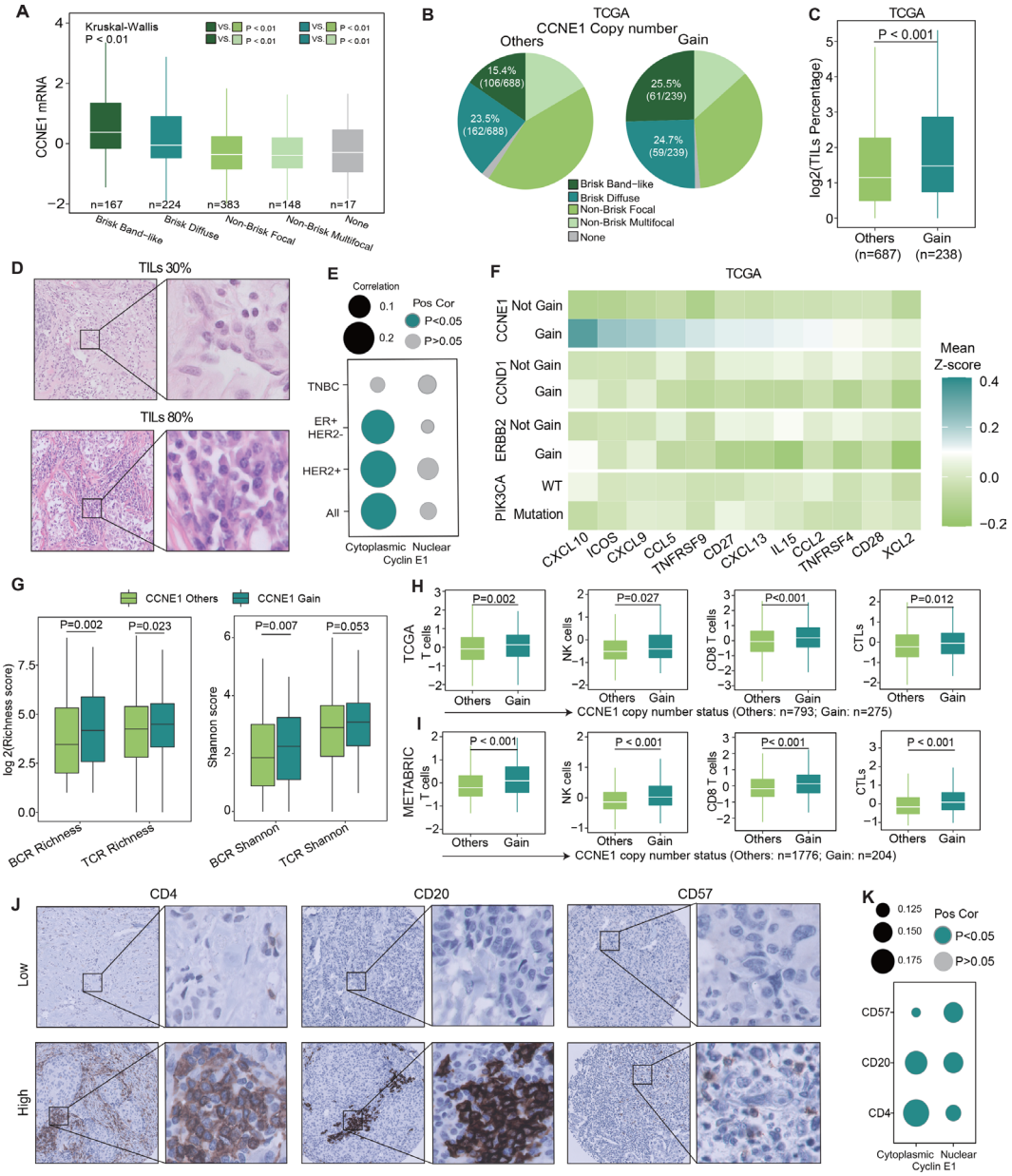

Figure 2 Cyclin E1 expression is correlated with immune cell infiltrate in the tumor microenvironment in patients with breast cancer. (A) The comparison of cyclin E1 mRNA expression in different spatial patterns of TILs in TCGA. Statistic test: Kruskal-Wallis test with the Dunn's multiple-comparison test was used for comparisons among groups. (B) The percentage of different TIL patterns between patients without or with CCNE1 copy number gain in the TCGA database. (C) Box plot showing TIL percentage between patients without or with CCNE1 copy number gain in the TCGA database. Statistic test: Wilcoxon rank-sum test. (D) Representative H&E staining images of breast cancer tissue with different percentages of TILs. (E) Correlation analysis between cyclin E1 expression and TIL levels in different breast cancer subtypes. The size of each circle represents the Spearman's correlation coefficient, and the color of the circle represents a positive or negative correlation with or without statistical significance. Statistic test: Spearman's correlation coefficient. (F) Heatmap showing the mean expression of immunomodulatory genes between different replication stress-related oncogene states. (G) The comparison of the richness and diversity score of TCR and BCR repertoire between patients without or with CCNE1 copy number gain in TCGA. Statistic test: Wilcoxon rank-sum test. (H–I) Box plot showing the immune cells' abundance (MCP-counter) between patients without or with CCNE1 copy number gain in TCGA (H) and METABRIC (I) database. Statistic test: Wilcoxon rank-sum test. (J) Representative images of CD4, CD20, and CD57 expression in patients with breast cancer's TMAs. (K) Correlation analysis between immune cell markers and cyclin E1 cytoplasm and nuclear in our TMAs. The size of each circle represents the Spearman's correlation coefficient, and the color of the circle represents a positive or negative correlation with or without statistical significance. Statistic test: Spearman's correlation coefficient. BCR, B-cell receptor; CTLs, Cytotoxic T Lymphocytes; ER+, estrogen receptor-positive; HER2, human epidermal growth factor receptor-2; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; MCP-counter, Microenvironment Cell Populations-counter; mRNA, messenger RNA; NK, natural killer; TCGA, The Cancer Genome Atlas; TCR, T-cell receptor; TILs, tumor infiltrating lymphocytes; TMAs, tissue microarrays; WT, wild type.

Consistent with our previous results, the *CCNE1* gain group showed an increased abundance of T cells, NK cells, $CD8^+$ T cells, and cytotoxic T lymphocytes (CTLs) using MCP-counter analysis (figure 2H,I). Moreover, ssGSEA analysis confirmed these results and specifically revealed the activation of B cells, as well as $CD4^+$ and $CD8^+$ T cells (online supplemental figure S4D–I). Besides, MCP-counter and ssGSEA analyses again revealed positive correlations between cyclin E1 mRNA expression and the different immune cell types, across both patient cohorts (online supplemental figure S4J,K). Subsequently, we IHC analyzed the presence of T cells, B cells, and NK cells markers in the breast cancer TME in our cohort and found that both cytoplasmic and nuclear cyclin E1 levels were positively correlated with the presence of T cells, B cells, and NK cells (figure 2J,K). Additionally, we also noticed that compared with patients with both low cytoplasmic and low nuclear cyclin E1 expression, cytoplasmic cyclin E1 high expression groups exhibited a higher number of CD4, but not CD20 and CD57 positive cells (online supplemental figure S5A–C). Taken together, *CCNE1* amplification and cytoplasmic cyclin E1 overexpression were associated with elevated immune cells in the TME in patients with breast cancer.

Cyclin E1 expression is associated with response to immunotherapy in patients with breast cancer

As increased immune cell infiltrate in the TME was previously associated with response to immunotherapy, we hypothesized that tumors harboring high levels of cyclin E1 also respond favorably to ICIs.[28 29] To assess the impact of cyclin E1 on immunotherapy response, we analyzed RNA-seq data and clinical data from three clinical trials evaluating immunotherapy response (online supplemental table S1). Within the I-SPY2 trial, a total of 73 and 69 patients with breast cancer received anti-PD-L1 (durvalumab) or anti-PD-1 (pembrolizumab), respectively, as part of their treatment (figure 3A). Notably, cyclin E1 mRNA levels in pretreatment tumors were significantly higher expressed in patients achieving pathologic complete response (pCR) in both the durvalumab and pembrolizumab arms (figure 3B,C). Similarly, the association of cyclin E1 with response to immunotherapy was in line with data from the IMvigor210 study, in which cyclin E1 mRNA expression was higher in patients with metastatic urothelial cancer who showed response to treatment (online supplemental figure S6A).

As both expression of immune checkpoint components and tumor mutation burden (TMB) emerged as important immunotherapy response predictors, we assessed the relationship between cyclin E1 expression or amplification, immune checkpoint expression, and TMB. Since the I-SPY2 trial did not provide TMB scores or PD-L1 protein expression levels, we first explored in the IMvigor210 cohort. TMB showed a significant positive correlation with cyclin E1 mRNA expression in the IMvigor210 cohort (online supplemental figure S6B). Moreover, the percentages of immune (IC) and tumor cells (TC) with high PD-L1 protein expression (≥5% stained on IC or TC) measured by IHC staining were increased in the cyclin E1-high expression group, compared with the low-cyclin E1 expression group (online supplemental figure S6C). In addition, we assessed whether cyclin E1 expression could improve the identification of potential responders in the IMvigor210 study, beyond PD-L1 expression or TMB. In both immune cell PD-L1 expression low (<1% stained (IC0) and ≥1% but <5% stained (IC1)) and high (≥5% stained (IC2+)) groups, the cyclin E1-high expression group patients all showed a higher response rate when compared with the cyclin E1-low expression group (online supplemental figure S6D). Moreover, in the group of tumor cells with low PD-L1 expression (<1% stained (TC0) and ≥1% but <5% stained (TC1)), the cyclin E1-high expression group showed a relatively higher response rate compared with the cyclin E1-low expression group (online supplemental figure S6D). However, this was not observed in the group with high tumor cell PD-L1 expression (≥5% stained (TC2+)) (online supplemental figure S6D). As for the TMB, in both TMB low and high groups, the cyclin E1-high expression group all showed a higher response rate when compared with the cyclin E1-low expression group (online supplemental figure S6D). In the TCGA and METABRIC breast cancer cohorts. Cyclin E1 mRNA expression was also positively correlated with TMB score (online supplemental figure S7A). In addition, the TMB score was higher in *CCNE1* gain tumors in the TCGA cohort, although the association was not observed in the METABRIC cohort (online supplemental figure S7A). Spearman's correlation analysis also showed that different immune checkpoint molecules were significantly positively correlated with cyclin E1 mRNA expression (online supplemental figure S7B) and showed higher expression in the *CCNE1* gain group in both cohorts (online supplemental figure S7C). Furthermore, we IHC analyzed the presence of PD-L1 in our patient cohort and observed a higher nuclear cyclin E1 expression level but not higher cytoplasmic cyclin E1 levels in the CPS-high patient group (online supplemental figure S7D–F).

CCNE1 is frequently amplified in various cancer types and is an established cause of replication stress.[30 31] Since cyclin E1 expression is dependent on cell cycle distribution, the mRNA expression may not completely reflect the *CCNE1* amplification status.[32] Therefore, we explored the role of *CCNE1* copy number status in immunotherapy response. Because the I-SPY2 study did not provide CNAs information, we obtained the transcriptional effects of CNAs for *CCNE1* using transcriptional adaptation to CNA (TACNA) profiling. In the I-SPY2 durvalumab arm, patients achieving pCR exhibited significantly higher *CCNE1* TACNA levels (figure 3D). Moreover, *CCNE1* TACNA levels showed a stronger association with response to treatment when compared with the mRNA expression of PD-L1 or PD-1 (figure 3D). As for the pembrolizumab arm, *CCNE1* TACNA level was also significantly higher in the pCR group, but showed a similar predictive power

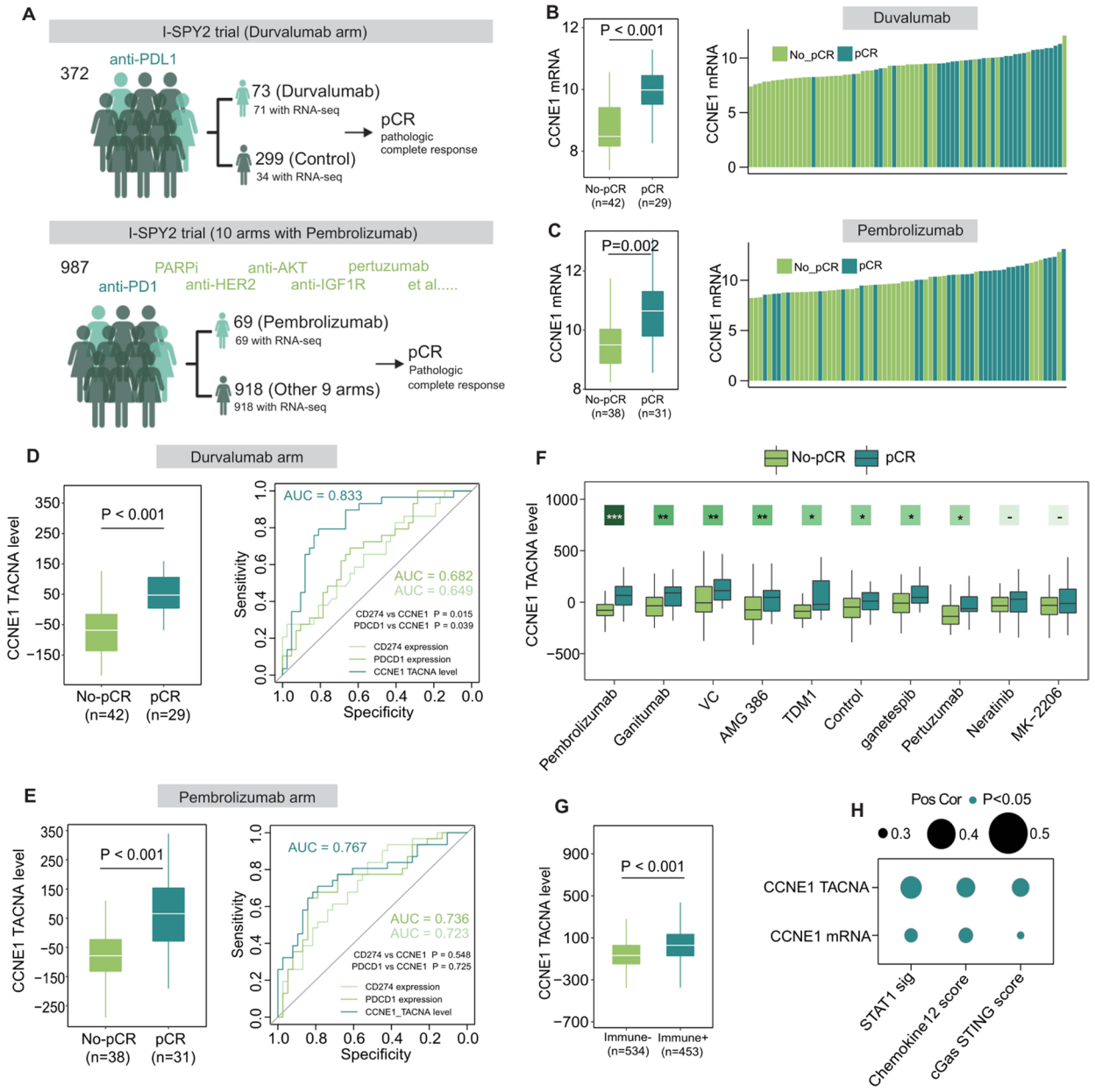

Figure 3 Cyclin E1 is associated with response to immunotherapy in patients with breast cancer. (A) Overview of two I-SPY2 clinical trials (created with BioRender.com). (B–C) Comparison of Cyclin E1 mRNA expression between no-pCR group and pCR group in the I-SPY2 durvalumab arm (B) and pembrolizumab arm (C). Statistic test: Wilcoxon rank-sum test. (D–E) Comparison of CCNE1 TACNA level between the no-pCR group and pCR group in two I-SPY2 trials. AUC of ROC curves comparing performance of CCNE1 TACNA level, PD-L1, and PD-1 mRNA expression level in two I-SPY2 trials. Statistic test: Wilcoxon rank-sum test. (F) CCNE1 TACNA level across 10 treatment arms in the I-SPY2 trial. Statistic test: Wilcoxon rank-sum test. (G) Comparison of CCNE1 TACNA level between immune$^-$ and immune$^+$ patients in the I-SPY2 trial. Statistic test: Wilcoxon rank-sum test. (H) Spearman's correlation analysis between cyclin E1 mRNA, CCNE1 TACNA level, and interferon-related signaling pathways in the I-SPY2 trial. The size of each circle represents the Spearman's correlation coefficient, and the color of the circle represents a positive or negative correlation with or without statistical significance. Statistic test: Spearman's correlation coefficient. AUC, area under the curve; mRNA, messenger RNA; pCR, pathologic complete response; PD-1, programmed death 1; PD-L1, programmed death ligand 1; ROC, receiver operating characteristic; RNA-seq, RNA sequencing; TACNA, transcriptional adaptation to copy number alteration profiling.

compared with the mRNA expression of PD-L1 and PD-1 (figure 3E). *CCNE1* TACNA levels were also significantly higher in patients who showed pCR in different treatment arms in the I-SPY2 trial, although the pembrolizumab treatment arm showing the most striking difference (figure 3F). Within the I-SPY2 trial, patients were also classified into immune-positive (immune$^+$) and immune-inactive (immune$^-$) phenotypes, with immune$^+$ patients being more likely to respond to immunotherapy. In line with our findings, immune$^+$ patients also showed significantly higher *CCNE1* TACNA levels (figure 3G). Based on our earlier observation that IFN-stimulated genes were

higher expressed in tumors with *CCNE1* copy number gain, we explored the relation between cyclin E1 and immune-related pathways in the I-SPY2 trial. Both cyclin E1 mRNA and TACNA level were positively correlated with the expression of immune-related signaling pathways (figure 3H). Furthermore, the predictive ability of two commonly used immune-related signatures, IFN-γ and the expanded Immune gene signature, was explored in the I-SPY2 trial. These two signatures both showed statistically significant associations with improved clinical outcomes in both pembrolizumab (online supplemental figure S8A,B and E,F) and durvalumab arms (online supplemental figure S8I,J and M,N). In the patients with hormone receptor-positive (HR+) and TNBC, both of these gene expression signatures were still significantly associated with patients' response to the pembrolizumab treatment (online supplemental figure S8C,D and G,H). However, whereas HR+ patients showed a statistically significant correlation between these signatures and durvalumab treatment response, this was not observed in patients with TNBCs (online supplemental figure S8K,L and O,P). Consistent with the results in the I-SPY2 trial, the Gene Set Enrichment Analysis enrichment analysis showed that in both TCGA and METABRIC cohorts, patients with breast cancer with high cyclin E1 mRNA expression were highly involved with IFN pathways (figure 4A–C; online supplemental figure 9A–C). Besides, the *CCNE1* copy number gain group also showed a higher enrichment score of these immune-related pathways in both databases (online supplemental figure S9D,E). Taken together, the results from three immunotherapy cohorts and publicly available databases demonstrated that cyclin E1 expression was associated with a better clinical response to immunotherapy treatment, with its potential underlying mechanisms involving IFN signaling.

Cyclin E1 overexpression induces micronuclei and activates an innate immune response

Building on our initial observation that cyclin E1 expression is related to immune cell abundance in the TME, we tried to elucidate the mechanisms through which cyclin E1 influenced antitumor immunity. Given that cyclin E1 overexpression leads to an increase in DNA lesions and triggers aberrant mitosis, activation of the cytoplasmic DNA sensor cGAS could link DNA damage in *CCNE1*-amplified cells to IFN pathway activation.[33 34] To test this hypothesis, we first calculated a previously described cGAS-STING activation score in samples of the TCGA and METABRIC cohorts.[35 36] The cGAS-STING scores were positively correlated with cyclin E1 mRNA expression and highly elevated in the *CCNE1* copy gain groups in both cohorts (online supplemental figure S9F,G). Subsequently, we overexpressed cyclin E1 in HCC1806 and HCC38 TNBC cell lines (figure 4D; Online supplemental figure S10A). Immunofluorescence microscopy analysis revealed significantly higher numbers of cGAS-positive micronuclei in both HCC1806 and HCC38 cell lines on cyclin E1 overexpression (figure 4E,F; online supplemental figure S10B,C). In line with this finding, cyclin E1-overexpressing cells expressed significantly higher mRNA levels of innate immune-related genes, including CXCL9, CXCL11, and IFN-β in HCC1806 cells (figure 4G). Also, in HCC38 cells, we observed a significantly higher expression of CXCL11 on cyclin E1 overexpression, whereas upregulation of CXCL9 and IFN-β upregulation was not significant (online supplemental figure S10D). Out of all cytokines measured, CXCL10 was not increased in either HCC1806 or HCC38 cells on cyclin E1 overexpression (figure 4G; online supplemental figure S10D). Subsequently, we IHC analyzed CXCL11 expression in our patient cohort and found that patients with both high cytoplasmic and high nuclear CXCL11 expression showed higher cyclin E1 expression compared with patients with both low cytoplasmic and low nuclear CXCL11 expression (online supplemental figure S10E–G). The transcription factors IRF3 and STAT1 are key components in the type I IFN pathway and regulate cytokine expression.[37] In line with this notion, both pIRF3 and pSTAT1 levels were increased on cyclin E1 overexpression in HCC1806 cells (figure 4H). Also, increased cell surface expression of PD-L1 was observed in HCC1806 cells on overexpression of cyclin E1 (figure 4I,J). Of note, IFN-γ treatment served as a positive control for PD-L1 induction in these experiments, and we observed that cyclin E1 overexpression could induce PD-L1 levels even further in IFN-γ-treated cells (figure 4I,J). Since cyclin E1-overexpressing cells secreted higher levels of innate immune-related cytokines, we further evaluated the effects of cyclin E1 expression on immune cell recruitment. Whereas downregulation of cyclin E1 overexpression using doxycycline-inducible shRNA only resulted in a minor and statistically not significant decrease of PMBC migration, cyclin E1 overexpression resulted in increased migration of PBMCs to tumor cells (online supplemental figure S10H,I; Figure 4K,L). Next, we tested whether immune cell migration is influenced by CXCL11. Addition of a CXCL11-blocking antibody resulted in decreased PBMC migration (figure 4L). To characterize the immune cell subsets that migrated towards tumor cells, flow cytometry was performed (online supplemental figure S11A,B). Flow cytometry analysis revealed an increase in CD8 T-cell percentage (online supplemental figure S11C). Additionally, an increase in the percentage of natural killer T (NKT) cells was observed, although overall only a small amount of NKT cells was measured (online supplemental figure S11C). Combined, these data suggest that cyclin E1 overexpression resulted in micronuclei formation and activation of the innate immunity, resulting in increased immune cell migration (online supplemental figure S11D).

DISCUSSION

In this study, we explored the relation of cyclin E1 expression with immune infiltrates in the TME and the association between cyclin E1 expression levels and response

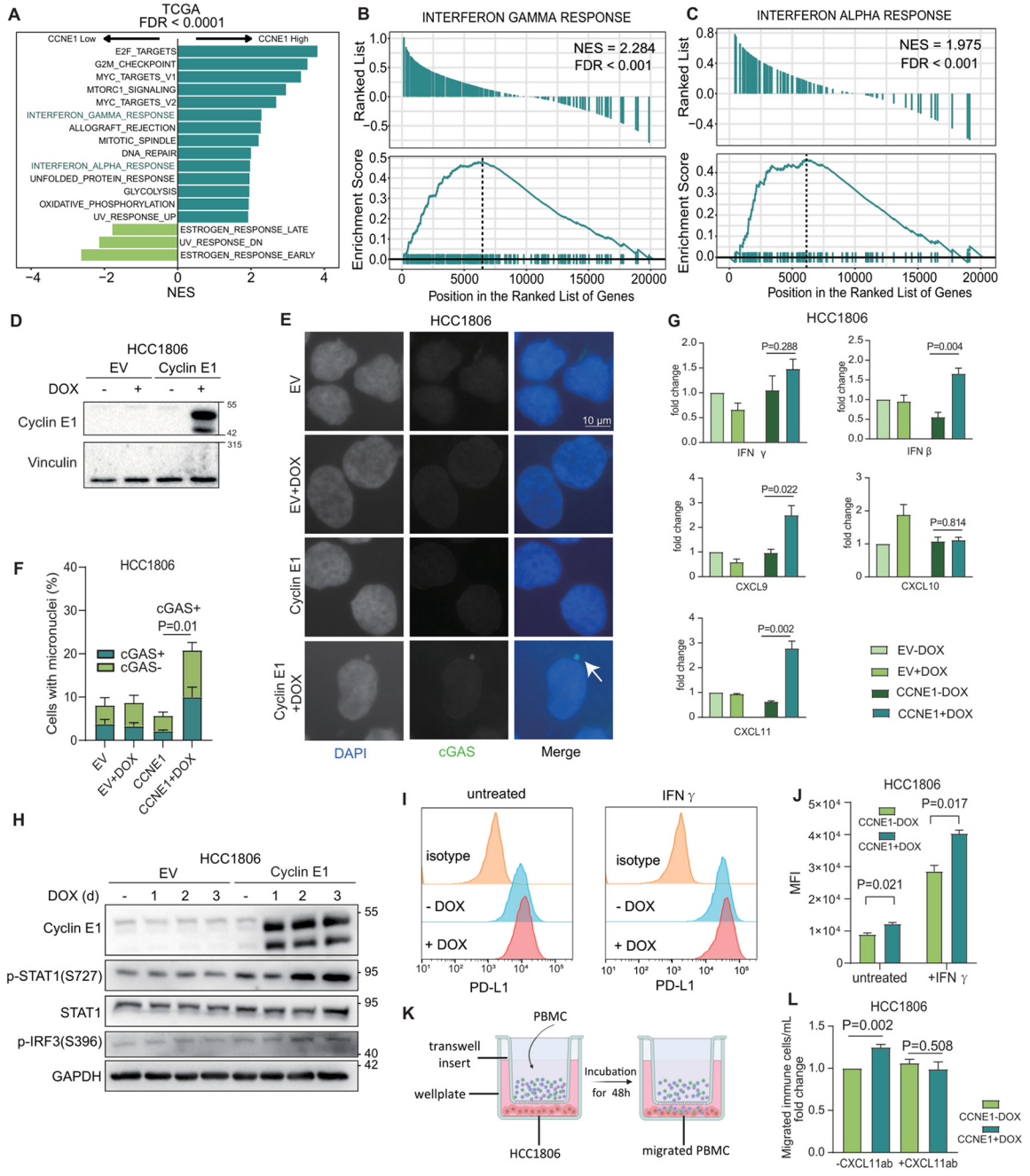

Figure 4 Cyclin E1 overexpression induced micronuclei and activates an innate immune response. (A) GSEA enrichment results with NES of top enriched biological pathways in the TCGA cohort (black: pathways with statistical significance; green: IFN-related pathways). Statistic test: the NES for the significantly enriched gene sets (FDR<0.05) are presented in the bar plot. (B–C) Enrichment plots for the two significant pathways that are related to IFN in the TCGA cohort. (D) Immunoblots showing the cyclin E1 overexpression in HCC1806 cells. (E) Representative images of HCC1806 cells±doxycycline (1 μg/mL) for 2 days. Cells were stained with anti-cGAS and DAPI. (F) Quantification of cGAS-positive micronuclei in HCC1806 cells as described in (E). At least 30 cells were counted per condition per replicate. Lines and error bars indicate the mean±SEM of 5 independent experiments. Statistic test: unpaired two-tailed t-test. (G) The expression of IFN-related genes was analyzed by RT-qPCR in HCC1806 cells. Cells were treated with doxycycline (1 μg/mL) for 2 days. Lines and error bars indicate the mean±SEM of 3 independent experiments. Statistic test: unpaired two-tailed t-test. (H) Immunoblotting was performed for cyclin E1, STAT1, pSTAT1, and p-IRF3 in HCC1806 cells. (I–J) Typical histogram and quantification of MFI of PD-L1 in HCC1806 cells±doxycycline (1 μg/mL, 4 days)±IFN-γ (100 ng/mL, 24 hours). Lines and error bars indicate the mean±SEM of 4 independent experiments. Statistic test: paired two-tailed t-test. (K) Schematic overview of the transwell assay (created with BioRender.com). (L) Migration of human PBMCs towards tumor cell conditioned media from HCC1806 cells. Lines and error bars indicate the mean±SEM of 3 independent experiments. Statistic test: unpaired two-tailed t-test. cGAS, cyclic GMP-AMP synthase; DAPI, 4′,6-diamidino-2-phenylindole; DOX, doxycycline; FDR, False discovery rate; false discovery rate; GSEA, Gene Set Enrichment Analysis; IFN, interferon; MFI, median fluorescence intensity; NES, normalized enrichment scores; PBMCs, peripheral blood mononuclear cells; PD-L1, programmed death ligand 1; TCGA, The Cancer Genome Atlas.

to immunotherapy in patients with breast cancer. We found that increased cyclin E1 expression was positively correlated with immune cell infiltration, immunotherapy biomarkers, and better response to immunotherapy in patients with breast cancer. Mechanistically, cyclin E1 overexpression in breast cancer cell lines resulted in activation of an innate immune response. Combined, the integration of immune-genomic, molecular, and clinical data points to cyclin E1 overexpression being a regulator of antitumor immunity in breast cancer.

CCNE1 amplification, as one of the major sources of replication stress in cancer cells, is known to induce premature entry into S phase, resulting in increased stress at replication forks and double strand breaks.[38] In line with these findings, a recent study highlighted that overexpression of cyclin E1, particularly in combination with cell division cycle 7-related protein kinase and ataxia telangiectasia and Rad3-related protein kinase inhibitor treatment, could escalate genomic instability and activate the innate immune response.[39] Concerning the clinical relevance of *CCNE1* amplification in cancers, previous studies have shown that *CCNE1* amplification is a prognostic factor related to poor survival in patients with cancer.[40] Interestingly, previous studies noted a trend toward increased infiltration of immune cells and a favorable response of *CCNE1* amplified patients to immunotherapy.[41–44] A phase I clinical trial combining CHK1 inhibition with anti-PD-L1 reported that three out of six patients with *CCNE1*-amplified high-grade serous ovarian cancer showed a partial response, and one patient maintained stable disease for more than 12 months.[41] Additionally, a trend towards improved survival after immunotherapy in patients with *CCNE1*-amplified gastric adenocarcinoma was observed.[42] The paradox between worse outcomes and improved response to immunotherapy treatment in *CCNE1*-amplified cancers highlights the complex role of cyclin E1 in cancer. While *CCNE1* amplification is seen in highly proliferating tumor types, which is associated with a favorable response to treatment initially, *CCNE1* amplification also causes genomic instability and the emergence of subclones that become treatment resistant, and may explain long-term poor survival outcomes.[45] In addition, differences in treatment strategies or data processing methods across studies may have influenced these analyses.[46] Clearly, the potential role and mechanisms of cyclin E1 in modulating the tumor-immune microenvironment and patient response to ICIs in patients with breast cancer still require further exploration.

One of the consequences of genomic instability is the leakage of DNA from the nucleus into the cytoplasm through micronuclei rupture.[12 13] Cytoplasmic DNA can subsequently trigger cytoplasmic DNA sensors, including cGAS. As a result, downstream pathways are activated, including a transcriptional program initiated by the IRF3 transcription factors, leading to a type I IFN response and JAK-STAT1 signaling.[37] Even though oncogene expression is a major cause of genomic instability, different oncogenes may induce a distinct immune landscape in cancer cells. For example, it has recently been reported that oncogenes like MYC or KRAS may have the ability to suppress immune TME through the IFN signaling.[47 48] In this study, we also demonstrated that breast tumors with cyclin E1 overexpression harbored the activation of innate immunity in breast cancer.

As increased TIL levels in TME were associated with favorable outcomes in immunotherapy, we further explored the relation between cyclin E1 expression and TIL levels. We found that cytoplasmic cyclin E1 expression in our patient cohort and *CCNE1* copy number gain in the TCGA cohort were both correlated with increased TIL levels in patients with breast cancer, whereas nuclear cyclin E1 expression was not. This difference may be attributed to bulk RNA-seq data in the TCGA data set, which may potentially mask the effects of distinct subcellular localizations. We also explored the relation between cyclin E1 expression and patients' response to ICIs. Through analysis of transcriptomic and clinical data from three clinical trials, we found that cyclin E1 overexpression was associated with better clinical response to ICIs treatment. A recent study reported that high cyclin E1 expression level had the potential as a biomarker to predict the use of combination CDK inhibitors/poly ADP-ribose polymerase (PARP) inhibitors in basal-like breast cancers.[49] Given that a number of preclinical studies suggested that the combination therapy of ICIs with PARP or CDK4/6 inhibitors showed response, cyclin E1 overexpression might serve as a potential biomarker to predict response to this combination therapy in breast cancer.[50]

Even though results from clinical trials suggest that cyclin E1 expression has potential as a predictor of response to ICIs treatment, this study has some limitations. First, we used a retrospective breast cancer cohort to verify the immune modulation role of cyclin E1, a prospective study will be required to further confirm the predictive ability of cyclin E1 to the ICIs treatment response. Second, we observed the pro-inflammatory effects of cyclin E1 overexpression using isogenic in vitro TNBC models. The use of additional models is required to reveal possible context-dependence. Additionally, exploration of the effects of cyclin E1 overexpression using in vivo studies can be used to solidify these findings and explore the efficacy of ICIs in *CCNE1*-amplified tumors. Third, the number of TILs in H&E slides in the public databases was measured through a machine learning method, which provides estimated TIL numbers with low immune cells and may deviate from manual scoring. Fourth, to align the criteria for clinical application of cyclin E1 as a patient selection criterion, it will be essential to establish standardized thresholds for cyclin E1 overexpression by integrating genetic and protein-level data across diverse cohorts.

In summary, our findings support that cyclin E1 overexpression or amplification correlates with antitumor immunity in patients with breast cancer and contributes to a better response to ICIs. Targeting cyclin E1, combined

with ICI, might offer new opportunities for therapeutic interventions in patients with breast cancer.

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Patient consent for publication Not applicable.

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