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Lab Note

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High phosphorylated cyclin-dependent kinase 2 expression indicates poor prognosis of luminal androgen receptor triple-negative breast cancer

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Triple-negative breast cancer (TNBC) is a highly aggressive breast cancer subtype characterized by a lack of estrogen receptorα (ER) and progesterone receptor (PR) expression and the absence of HER2 overexpression or amplification. TNBC's high heterogeneity in both molecular and clinical features hinders the development of effective treatments; thus, further molecular classification of TNBC is still needed. The luminal androgen receptor (LAR) subtype is the differentiated subtype of TNBC, displaying a luminal expression profile with clinical features that are distinct from other TNBC tumors, which leads to insensitivity to chemotherapy [1,2]. Therefore, studies on molecular targets in LAR subtype may provide potential therapeutic strategies for LAR TNBC. Due to higher expression levels of AR protein and the active state of the AR signaling pathway in LAR patients, hormone receptor blockade was first selected to investigate its treatment effect in LAR TNBC. However, only the modest benefit of AR inhibition has been observed in these patients. On the other hand, inhibition of cell cycle regulators generally works well in tumors with luminal genetic profiles, such as CDK4/6 inhibitors in ER/PR$^+$, HER2$^-$ breast cancer. LAR subtype tumors also display highly activated cell cycle signaling, with a high proportion of RB1 neutral and low CCNE1 transcriptional expression levels [3,4]. These findings prompted interest in exploring the role of CDK4/6 inhibitors in this subgroup. However, despite encouraging signs from preclinical studies, most advanced LAR patients progressed during treatment with anti-CDK4/6 therapy in a clinical setting [5].

In Rb-positive cancer cells, those with hyperactive cyclin E/CDK2 complex may bypass CDK4/6 inhibition [4,6]. LAR tumors retain RB1 [3], and CDK2 kinase activity in the LAR patient population is still not well characterized. In this study, we aimed to investigate the expression of CDK2 phosphorylated at Thr160 (pCDK2), which is indicative of the activated cyclin E/CDK2 complex, in LAR tissues using immunohistochemistry (IHC). TNBC tumors, especially the non-LAR TNBC basal and mesenchymal subtypes, are conventionally considered poor candidates for CDK4/6 inhibitors partially due to upregulated activity of the cyclin E/CDK2 complex, while ER-positive and/or PR-positive, HER2-negative breast cancer patients only truly benefit from CDK4/6 inhibitor therapy when they have relatively low CDK2 activity [4,7]. pCDK2 levels in patients from three groups of tumors (TNBC LAR, TNBC non-LAR and ER/PR-positive, HER2-negative) were also compared.

We collected clinical data and surgical specimens from a cohort of 634 early-stage breast cancer patients diagnosed from January 2010 to January 2012 at the First Hospital of China Medical University. Collection of the human specimens in this study was approved by the Human Research Ethics Committee of China Medical University (Permit Number: [2020]27). The enrollment criteria for the study included the following: (1) histologically confirmed diagnosis of invasive breast cancer, of no special type; (2) TNBC, defined as ER$^-$, PR$^-$, HER2$^-$ breast cancer. The cutoff values for ER positivity and PR positivity were 1% of positive tumor cells with nuclear staining. Positivity for HER2 was considered when IHC staining is over 3+ or with positive fluorescence in *in situ* hybridization (FISH); (3) a paraffin block with an available tumor tissue for AR and pCDK2 immunostaining, and (4) no sign of metastasis at diagnosis. Sections (5 μm thick) were prepared from formalin-fixed, paraffin-embedded breast cancer tissue specimen blocks and were dewaxed, rehydrated, blocked for endogenous peroxidase with 3% H_2O_2 solution, and boiled in citrate buffer for antigen retrieval. The sections were then incubated with 10% normal goat serum solution to block nonspecific binding, followed by incubation with a rabbit polyclonal antibody against p-CDK2 Thr160 (1:400; Abcam, Cambridge, UK) or a rabbit monoclonal antibody against AR (1:200; Abcam), followed by incubation with streptavidin-peroxidase-conjugated secondary antibodies (Fuzhou Maixin Biotech. Co., Ltd, Fuzhou,

China). The signals were visualized with diaminobenzidine, and the nuclei were counterstained with hematoxylin. Staining results were evaluated by two certified pathologists who were blinded to the clinical data. AR was considered positive in the case of nuclear staining in more than 10% of tumor cells. The level of pCDK2 was semi-quantitatively classified according to the immunoreactivity score (IRS), which was calculated from the result of the intensity score (0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining) multiplied by the distribution score (0% = 0, 1% to 10% = 1, 11% to 50% = 2, 51% to 80% = 3, 81% to 100% = 4). Nuclear staining and cytoplasmic staining were scored individually, with a separate determination of cytoplasmic IRS and nuclear IRS. Total IRS was calculated by adding cytoplasmic IRS and nuclear IRS (Figure 1).

To reduce differences among clinical characteristics between LAR patients and ER/PR-positive, HER2-negative patients, we conducted propensity score matching (PSM) analysis. Propensity scores were calculated using a logistic regression model for each LAR and ER/PR-positive, HER2-negative patient, and the following independent variables were included: age, tumor stage, lymph node status, and recurrence. The χ^2 test or one-way ANOVA test was used to examine the associations between the clinicopathological parameters and pCDK2 level, according to their nature. Differences in pCDK2 levels among the three cohorts were evaluated using the nonparametric Kruskal–Wallis test. Cutoff values of total, nuclear and cytoplasmic pCDK2 IRS scores were determined using X-tile 3.6.1 software (Yale University, New Haven, USA) [8] by analyzing the TNBC survival data. Using this approach, IRS scores of 0 to 4 were defined as low level for either total or cytoplasmic pCDK2, and IRS scores of 0 to 2 were defined as low level for nuclear pCDK2. Survival end points included disease-free survival (DFS) and overall survival (OS). Survival curves were estimated using the Kaplan–Meier method, and the log-rank test was used to compare differences between patients with different pCDK2 levels. Univariate and multivariate Cox proportional hazard models were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). All reported P values are two-sided, and the significance level was set at $P < 0.05$. All statistical analyses were performed using SPSS 22.0 software for Windows.

As shown in Supplementary Figure S1, there were 102 TNBC patients in our study population, and 16 patients were excluded because of conditions such as special types of breast cancer (Paget's disease or apocrine carcinoma) or ductal carcinoma *in situ*, yielding a total of 86 TNBC cases for further analysis between LAR and non-LAR TNBC in this study. After analyzing the intensity of AR expression using IHC, we classified 29 (33.7%) TNBC samples with more than 10% positively stained tumor cells into the LAR subtype, consistent with the FUSCC IHC-based TNBC subtype classification [9]. We next performed PSM analysis to generate the ER/PR-positive, HER2-negative cohort with similar clinical features to those in the LAR cohort.

The clinical characteristics of the TNBC non-LAR cohort, TNBC LAR cohort, and PSM ER/PR-positive, HER2-negative cohort are shown in Supplementary Table S1. Coincidentally, patient profiles of TNBC non-LAR cohorts were comparable to TNBC LAR and ER/PR-positive, HER2-negative cohorts with respect to age, tumor stage, lymph node stage and recurrence status ($P > 0.05$). High Ki-67 expression (≥20%) was more frequently observed in TNBC non-LAR patients than in the TNBC LAR cohort and ER/PR-positive, HER2-negative cohort ($P = 0.001$), which is in line with observations from other groups. No significant difference was observed in Ki-67 status between the LAR cohort and the ER/PR-positive, HER2-negative cohort ($P = 0.458$). In the ER/PR-positive, HER2-negative cohort, 82.76% of patients were ER-positive (> 10%), and 17.24%

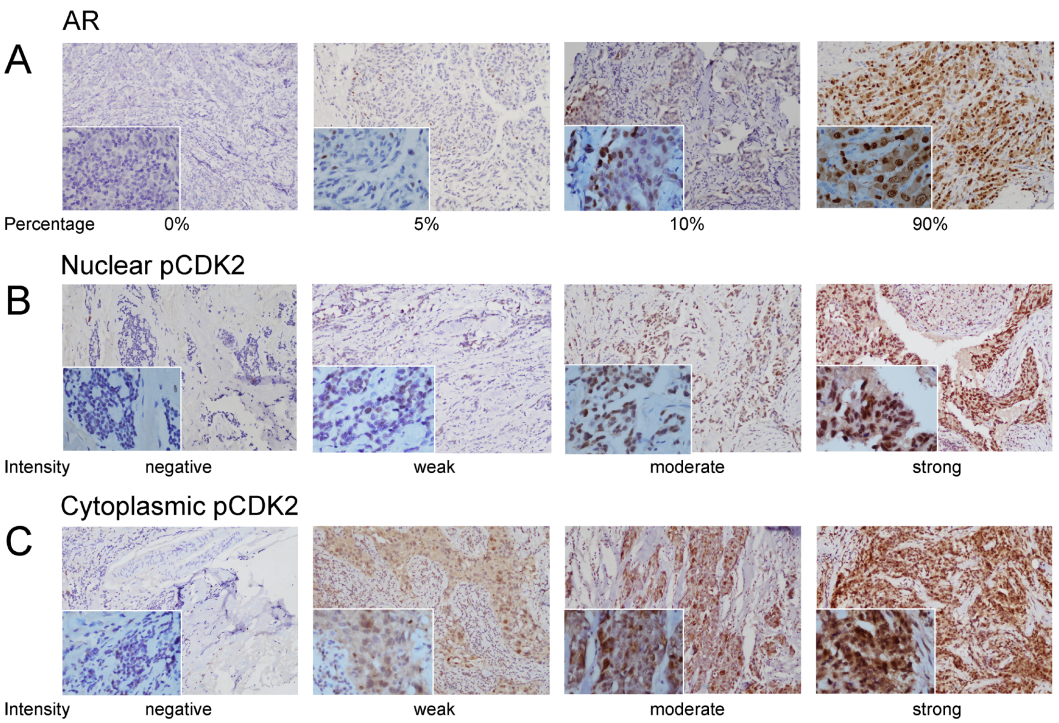
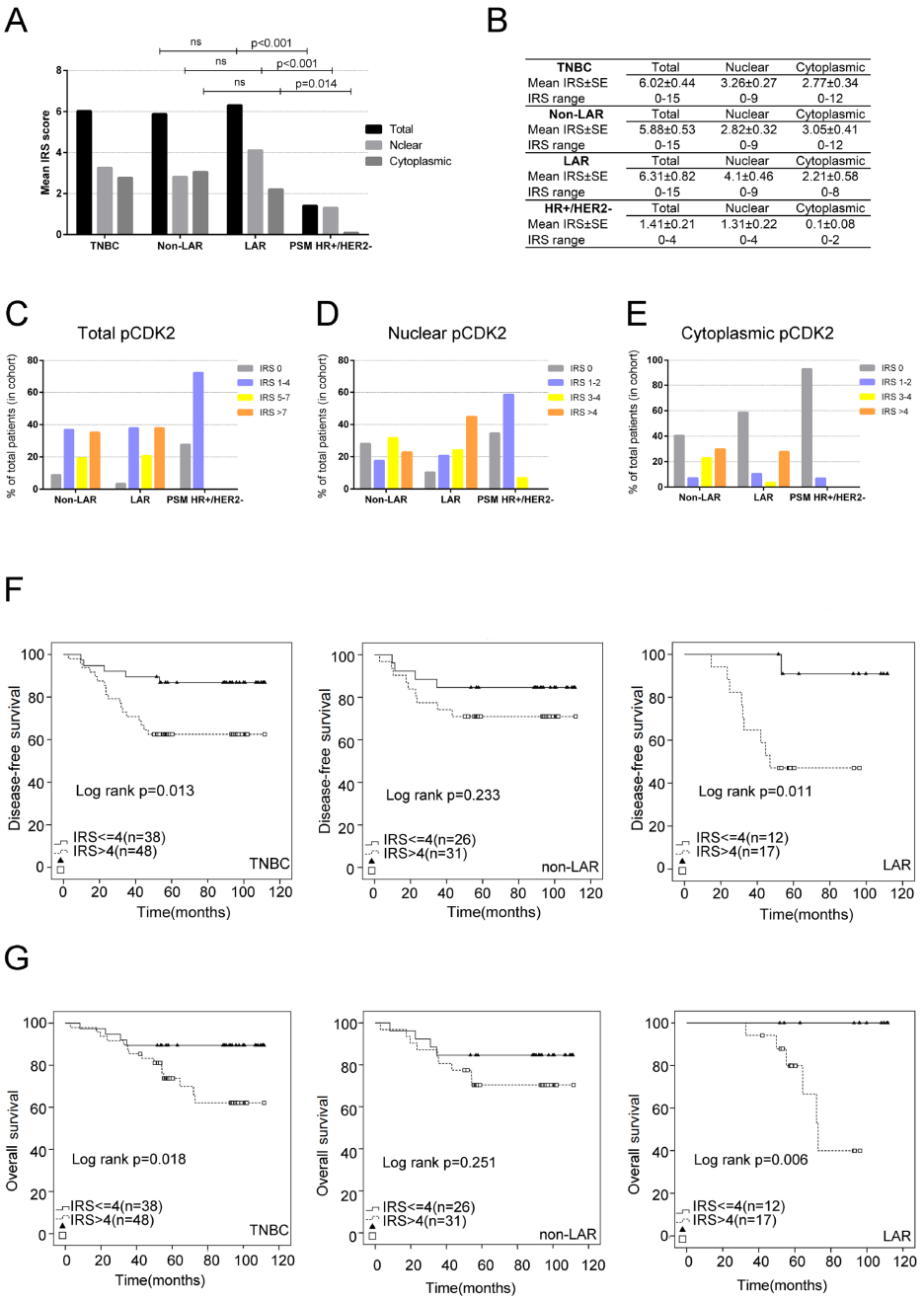


Figure 1. Representative images of immunohistochemical staining for AR and pCDK2 in breast cancer tissues (A) Different expression level of AR as indicated in breast cancer tissues. (B,C) Different intensities of nuclear pCDK2 (B) and cytoplasmic pCDK2 (C) in breast cancer tissues (×100, insert ×400).

of patients were ER-low-positive (1%–10%).

IHC were performed to examine pCDK2 expression in TNBC LAR cohort, TNBC non-LAR cohort and ER/PR-positive, HER2-negative cohort. The results demonstrated that pCDK2 expression was positive (total IRS > 0) in 91.2% of non-LAR TNBC tissues, 96.6% of LAR TNBC tissues, and 72.4% of PSM ER/PR-positive, HER2-negative tissues. Positive nuclear pCDK2 (nuclear IRS > 0) immunostaining was detected in 71.9% of non-LAR TNBC tissues, 89.7% of LAR TNBC tissues, and 65.5% of PSM ER/PR-positive, HER2-negative tissues. Positive cytoplasmic pCDK2 (cytoplasmic IRS > 0) immunostaining was detected in 59.6% of non-LAR TNBC tissues, 41.4% of LAR TNBC tissues, and 6.9% of PSM ER/PR-positive, HER2-negative tissues. Next, we compared pCDK2 levels among non-LAR TNBC, LAR TNBC tissues, and PSM ER/PR-positive, HER2-negative tissues (Figure 2). Both nuclear and cytoplasmic expression of pCDK2 in LAR patients was significantly elevated compared to those in ER/PR-positive, HER2-negative tissues (nuclear mean IRS score: 4.1 vs 1.3, Kruskal–Wallis test $P < 0.001$; cytoplasmic mean IRS score: 2.21 vs 0.1, Kruskal–Wallis test $P = 0.014$), of course, for total expression of pCDK2 (total mean



TNBC	Total	Nuclear	Cytoplasmic
Mean IRS±SE	6.02±0.44	3.26±0.27	2.77±0.34
IRS range	0-15	0-9	0-12
Non-LAR	Total	Nuclear	Cytoplasmic
Mean IRS±SE	5.88±0.53	2.82±0.32	3.05±0.41
IRS range	0-15	0-9	0-12
LAR	Total	Nuclear	Cytoplasmic
Mean IRS±SE	6.31±0.82	4.1±0.46	2.21±0.58
IRS range	0-15	0-9	0-8
HR+/HER2-	Total	Nuclear	Cytoplasmic
Mean IRS±SE	1.41±0.21	1.31±0.22	0.1±0.08
IRS range	0-4	0-4	0-2

Figure 2. Comparison of pCDK2 levels among TNBC non-LAR tissues, TNBC LAR tissues and PSM ER/PR-positive, HER2-negative tissues and the cooperation of total pCDK2 expression with survival in TNBC patients (A,B) Mean IRS scores of total, nuclear and cytoplasmic pCDK2 in different cohorts. ns: no significant. (C–E) Percentage of various scales of total, nuclear and cytoplasmic pCDK2 IRS scores in different cohorts. Gray bars, blue bars, yellow bars and red bars indicate different staining IRS scores. (F,G) Kaplan–Meier analysis of DFS and OS according to total pCDK2 expression in the whole TNBC cohort, TNBC non-LAR cohort and TNBC LAR cohort. Statistical significance is shown as p-value from log-rank test.

IRS score: 6.31 vs 1.41, Kruskal–Wallis test $P < 0.001$). There was no significant difference in pCDK2 expression between TNBC LAR tissues and TNBC non-LAR tissues (nuclear mean IRS score: 4.1 vs 2.82; cytoplasmic mean IRS score: 2.21 vs 3.05; total mean IRS score: 6.31 vs 5.88, all Kruskal–Wallis test $P > 0.05$).

Next, we compared pCDK2 expression and clinicopathological characteristics at initial diagnosis in the TNBC cohort. In the TNBC LAR cohort, high expression of total pCDK2 was significantly associated with recurrence ($P = 0.019$). Although not statistically significant, LAR tissues exhibited relatively higher levels of total pCDK2 in the high-level Ki-67 group ($P = 0.061$; Supplementary Table S2). In the TNBC non-LAR cohort, high expression of total pCDK2 was more common in relatively young patients (mean age: 51.77 years old vs 45.68 years old, $P = 0.011$). We also observed a positive association of borderline significance ($P = 0.052$) between nuclear pCDK2 level and tumor size in the non-LAR cohort (Supplementary Table S3).

Finally, we explored the association between pCDK2 expression and survival in TNBC cohort. The median follow-up time of all TNBC patients was 93.80 months [95% confidence interval (CI), 91.10–96.50 months], 23 (26.7%) patients experienced recurrence, and 19 (22.1%) patients experienced a death event. The mean survival times of all TNBC, non-LAR patients and LAR patients were 94.45, 92.71, and 96.79 months, respectively. TNBC patients with high levels of total pCDK2 exhibited worse DFS (log rank $P = 0.013$; Figure 2F) and OS (log rank $p = 0.018$; Figure 2G) in the Kaplan–Meier survival analysis. Stratifying TNBC patients according to AR status revealed that total pCDK2 was a significant predictor of shorter DFS (log rank $P = 0.011$; Figure 2F) and OS (log rank $P = 0.006$; Figure 2G) in TNBC LAR patients but not in TNBC non-LAR patients. In addition to total pCDK2 expression, survival analysis was also performed in parallel for nuclear and cytoplasmic pCDK2 expression. High nuclear pCDK2 expression was associated with worse OS in LAR patients (log rank $P = 0.034$; Supplementary Figure S2B). However, no significant differences in DFS or OS were observed between the nuclear/cytoplasmic pCDK2-high and pCDK2-low patients in our study (Supplementary Figure S2). Since Kaplan–Meier survival curves showed that the prognostic effect of total pCDK2 on survival was more obvious in LAR patients, the Cox proportional hazards model was used to assess the independent predictive role of total pCDK2 expression for disease recurrence in LAR patients (there were not enough events to access this information for OS in LAR patients). The univariable Cox proportional hazards model demonstrated that T stage (T1 vs T2-4, HR = 4.67, 95% CI 0.59–36.90, $P = 0.144$) and total pCDK2 expression (IRS $<= 4$ vs > 4, HR = 9.15, 95% CI 1.15–72.65, $P = 0.036$) were potentially associated with DFS in LAR patients and were considered eligible for multivariate proportional hazard models under the standard of a P value less than 0.15 (Supplementary Table S4). In multivariate analysis, total pCDK2 expression maintained an independent prognostic role (IRS $<= 4$ vs > 4, HR = 8.16, 95% CI 1.02–65.27, $P = 0.048$).

Both nuclear and cytoplasmic pCDK2 in LAR tumors were significantly elevated compared with those in ER/PR-positive, HER2-negative tissues. Although high level of pCDK2 was observed in LAR and non-LAR TNBC cohorts, the pCDK2 level was positively correlated with the poor prognosis only in the LAR cohort. Considering that Rb is one of the key CDK2 substrates in regulation of cell cycle process, this difference might be, if not all, but at least partially due to the high rate (58%) of RB1 loss/deletion in non-LAR TNBC patients [3]. It has been reported that androgen-dependent prostate cancer cells exhibit increased CDK2 kinase activity in response to androgen stimulation. LAR tumors with high level expression of AR also promote cell proliferation after androgen stimulation. In LAR subtype TNBC, whether CDK2 is induced by AR signaling pathway, thereby increasing CDK2 activity; the functions of nuclear and cytoplasmic pCDK2 in the specific context of LAR TNBC still need to be investigated.

In conclusion, our findings indicate that LAR TNBC displays high level of nuclear and cytoplasmic CDK2 in the active state, suggesting that total pCDK2 expression may serve as an important prognostic indicator in LAR patients. CDK2 inhibitors, as promising targets for therapy in LAR TNBC, are worth further exploration.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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