Targeting PKMYT1 kinase is an effective treatment strategy in Triple Negative Breast Cancers with low molecular weight cyclin E (LMW-E) expression

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Abstract

Cyclin E, is post-translationally modified by neutrophil elastase mediated proteolytic cleavage to generate the low molecular weight isoforms of cyclin E (LMW-E) that are detected in various human cancers. We previously reported that 70% of triple negative breast cancers (TNBC) examined overexpress LMW-E, and these patients have a poor prognosis. Expression of LMW-E promotes genomic instability by causing DNA replication stress. PKMYT1, prevents premature mitotic entry by catalyzing CDK1 phosphorylation at T14, essential for preventing DNA damage and cell death when cyclin E, including LMW-E, is overexpressed. In this study, we tested the hypothesis that LMW-E positive status can be used as a biomarker of response in selecting TNBC patients who are likely to respond to RP-6306, a first in-class and selective inhibitor of PKMYT1 kinase.

Assessment of pre-treatment breast biopsies from TNBC patients (n=36) enrolled in a neoadjuvant chemotherapy prospective study for LMW-E and CDK1-pT14 revealed significant positive correlation between these two proteins. Furthermore, positivity of both biomarkers was associated with lack of pathologic complete response (pCR) to neoadjuvant chemotherapy. We next examined the mechanism of response to RP-6306 *in vitro* and *in vivo* using TNBC cell lines, patient-derived xenograft (PDX) models and transgenic mouse mammary tumor virus (MMTV) models expressing human LMW-E (hLMW-E). *In vitro* results using 7 different TNBC cell lines, revealed that high LMW-E levels are significantly predictive of response to RP-6306 (R^2=0.78, p= 0.008), while LMW-E knockdown resulted in a 7X increase in IC50 values of RP-6306 (p>0.001). In high LMW-E cells, treatment with RP-6306 resulted in significant (i) accumulation of sub-G1 and polyploid cell population, (ii) apoptosis, (iii) accumulation of chromosomal breakage, (iv) increased DNA damage and (v) premature mitotic entry. Treatment of both breast cancer PDX models and hLMW-E transgenic tumors with RP-6306 revealed that only in animals with high LMW-E tumors, treatment results in significant decrease in tumor volume. However, RP-6306 was ineffective in reducing tumor volume in low cyclin E *in vivo* models. Immunohistochemical analysis revealed that *in vivo* efficacy of RP-6306 (in both PDX and transgenic models) was concomitant with increase in γ-H2AX and decrease in CDK1-pT14 and Ki67.

Collectively, our results show that overexpression of LMW-E and CDK1-pT14 in TNBC can be used to stratify patients whose tumors are likely to respond to RP-6306. Mechanistically, LMW-E overexpressing TNBC cells activate CDK1 (in vitro and in vivo) to accelerate premature mitotic entry, leading to DNA damage and apoptosis.

Introduction

Cyclin E, a regulatory subunit of CDK2, plays a critical role in the initiation of DNA synthesis at the G1/S stage and mediates the entry and progression of cells into S phase[1]. In cells with amplified cyclin E gene, synthetic lethal screening shows strong dependency on PKMYT1, a CDK1 inhibitory protein kinase that predominantly phosphorylates the threonine-14 on CDK1[2].

LMW-E is a truncated isoform of cyclin E that is found in tumor cells and tissues but not adjacent normal tissues[1]. LMW-E overexpression induces increased genomic instability, replication stress tolerance and increased DNA damage repair, which are associated with LMW-E driven breast tumors[3]. In this study, we initially observed positive correlation among LMW-E, CDK1 pT14 and clinical response following neo-adjuvant chemotherapy in TNBC patients treated with neoadjuvant chemotherapy and hypothesized that ***LMW-E may predict response to the PKMYT1 inhibitor (RP-6306). We tested this hypothesis by targeting the PKMYT1 kinase with RP-6306 using in vivo and in vitro model systems with or without expression of LMW-E.***

References:

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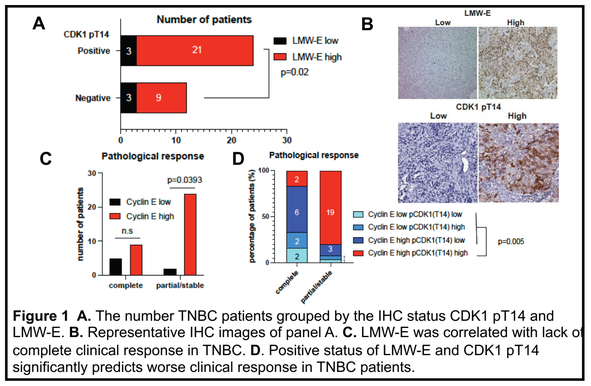

Figure 1 A. The number TNBC patients grouped by the IHC status CDK1 pT14 and LMW-E. **B.** Representative IHC images of panel A. **C.** LMW-E was correlated with lack of complete clinical response in TNBC. **D**. Positive status of LMW-E and CDK1 pT14 significantly predicts worse clinical response in TNBC patients.

LMW-E and CDK1 pT14 correlate with a lack of response in TNBC

We analyzed a cohort of 36 TNBC patients treated with neo-adjuvant chemotherapy at MDACC and found that LMW-E is co-overexpressed with CDK1 pT14 (p=0.02, **Figure 1A**). Both LMW-E (**Figure 1B, 1C**) and CDK1 pT14 (data not shown) alone were significantly correlated with a lack of pathological complete response (pCR, p=0.0067 for CDK1 pT14, and p=0.0393 for LMW-E). Patients with high levels of both LMW-E and CDK1 pT14 experience a lack of pCR to neoadjuvant chemotherapy (p=0.005, **Figure 1D**). We predict that patients whose tumors have high levels of LMW-E and CDK1pT14 are likely to respond to a PKMYT1 inhibitor.

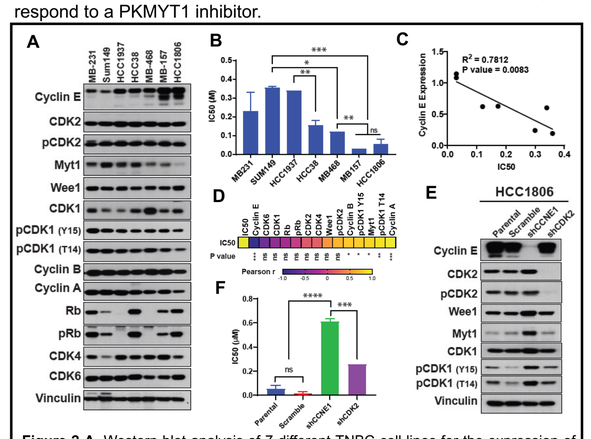

Figure 2 A. Western blot analysis of 7 different TNBC cell lines for the expression of Cyclin E (LMW-E) and key cell cycle proteins. **B.** RP-6306 IC50s in cell lines shown in panel A. Cell lines with high LMW-E (MDA-MB-157 and HCC1806) showed significantly lower IC50s of RP-6306. **C.** High LMW-E levels are significantly predictive of response to RP-6306 (R^2=0.78, p= 0.008). **D.** Summary of p values showing correlation between indicated protein expressions and RP-6306 IC50s. **E.** Establishment of cyclin E and CDK2 knock-down stable cell lines in HCC1806 cells (high endogenous LMW-E). **F.** Downregulation of LMW-E in HCC1806 cells significantly increased RP-6306 IC50.

LMW-E predicts response to protein kinase PKMYT1 inhibitor RP-6306

To investigate the role of LMW-E in the cellular response to PKMYT1 inhibitor RP-6306, we analyzed the IC50 values of RP-6306 in seven TNBC cell lines with different LMW-E expression levels (**Figure 2A**) . Our results suggested that TNBC cells endogenously showing higher LMW-E levels (such as HCC1806 and MDA-MB-157) exhibited significantly lower RP-6306 IC50s compared to cells without endogenous LMW-E expression (such as MDA-MB-231 and SUM149, **Figure 2B**). RP-6306 IC50 was negatively associated with the densitometry of LMW-E western-blotting but not FL-cycE (**Figure 2C, 2D** and data not shown). In HCC1806 cells that endogenously express high LMW-E, knock-down of CCNE1 but not CDK2 significantly increased IC50 of RP-6306, suggesting LMW-E may be required for sensitivity to PKMYT1 inhibition in breast cancer (**Figure 2E and 2F**).

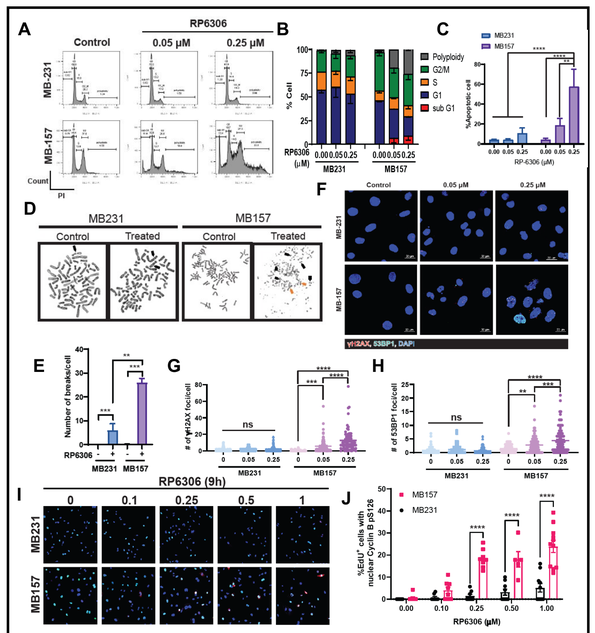

Figure 3A. RP-6306 treatment caused increased accumulation of sub-G1 and polyploid cell populations in LMW-E high TNBC cell line MDA-MB-157 but not LMW-E low TNBC cell line MDA-MB-231. **B.** Quantitation of cell cycle distributions of panel A. **C.** RP-6306 treatment caused increased apoptosis (Annexin V/PI staining) in MDA-MB-157 but not MDA-MB-231. **D.** Metaphase spread analysis for chromosomal aberrations in MDA-MB-157 and MDA-MB-231 with or without RP-6306 treatment. **E.** Quantitation of chromosomal breakages in panel D. **F.** DNA damage analysis for γH2AX and 53BP1 foci by IF in MDA-MB-157 and MDA-MB-231 with or without RP-6306 treatment. **G and H.** Quantitation of γH2AX (panel G) and 53BP1(panel H) foci in panel F. **I.** IF analysis for EdU and Cyclin B pS126 in MDA-MB-157 and MDA-MB-231 with or without RP-6306 treatment. **J.** Quantitation of EdU and Cyclin B pS126 positive cells in panel I.

LMW-E regulates the effect of RP-6306 in breast cancer cells

In LMW-E low TNBC cell line MDA-MB-231 and LMW-E high TNBC cell line MDA-MB-157, we observed different cellular characteristics in response to PKMYT1 inhibition. RP-6306 treatment caused increased accumulation of sub-G1 and polyploid cell populations in MDA-MB-157 but not MDA-MB-231 (**Figure 3 A and B**). MDA-MB-157 also showed higher apoptosis (**Figure 3C**) and accumulation of chromosomal breakages upon treatment with RP-6306 (**Figure 3D and E**). Immunofluorescent analysis using DNA damage markers showed increased DNA damage marker γH2AX and 53BP1 in MDA-MB-157 but not MDA-MB-231 cells (**Figure 3F-H**); and mitotic marker Cyclin B pS126 increased in MDA-MB-157 cells with DNA replication marker EdU, indicating premature mitotic entry induced by RP-6306 (**Figure 3I and J**).

RP-6306 shows selective response in LMW-E high *in vivo* models

To determine the effect of PKMYT1 inhibitor in LMW-E overexpression in the *in vivo* models of breast cancer, we transplanted LMW-E driven mouse mammary tumors from LMW-E transgenic mice into FVB mice and treated them with PKMYT1 inhibitor RP-6306 (10mg/kg or 20mg/kg) or vehicle control (BID, continuous dosing). RP-6306 treatment significantly reduced tumor volume (**Figure 4A**). IHC analysis confirmed decreased CDK1 pT14 and increased γH2AX by RP-6306 treatment (**Figure B-D**).

We also generated patient derived xenografts (PDX) models of TNBC with either high LMW-E (BCX070) or low LMW-E (XC5172013) expression. We transplanted these PDXs into nude mice and subjected them to treatment with 20mg/kg PKMYT1 inhibitor RP-6306 or vehicle (BID, continuous dosing. In LMW-E high PDX BCX070, treatment of RP-6306 significantly inhibited tumor growth (**Figure 4E**). In LMW-E negative PDX XC5172013, we did not observe a significant inhibitory effect of RP-6306 as compared to vehicle treated arm (**Figure 4F**).

As expected, RP-6306 significantly reduced CDK1 pT14 in both high and low LMW-E PDXs, suggesting RP-6306 successfully reached its target (**Figure 4G-I**). Increased DNA damage was only observed in LMW-E high PDX BCX070 (**Figure 4G and J**), but not LMW-E negative PDX XC5172013 (**Figure 4G and K**). Consistently, proliferation maker Ki67 was only inhibited by RP-6306 in LMW-E high PDX BCX070 (**Figure 4G and L**), but not in LMW-E negative PDX XC5172013 (**Figure 4G and M**).

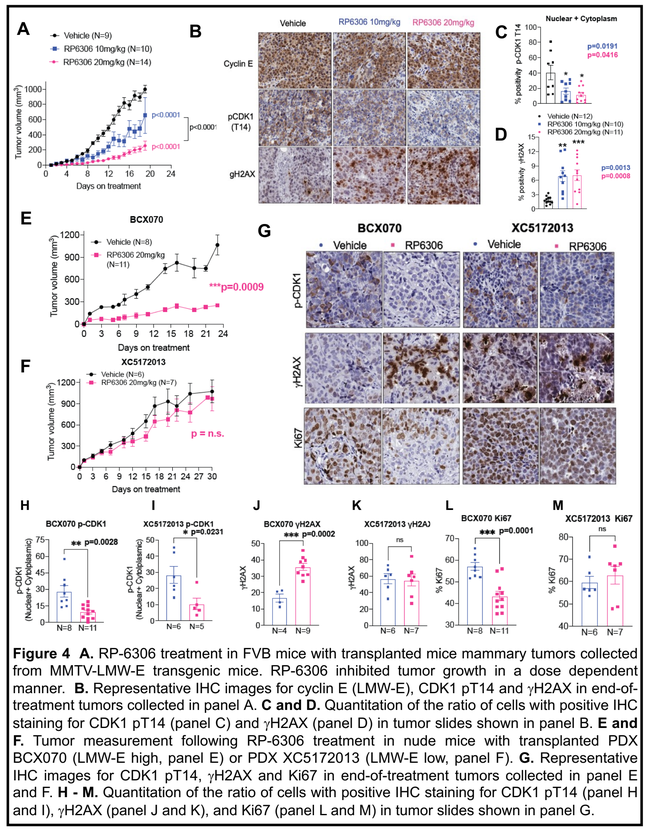

Figure 4 A. RP-6306 treatment in FVB mice with transplanted mice mammary tumors collected from MMTV-LMW-E transgenic mice. RP-6306 inhibited tumor growth in a dose dependent manner. **B.** Representative IHC images for cyclin E (LMW-E), CDK1 pT14 and γH2AX in end-of-treatment tumors collected in panel A. **C and D.** Quantitation of the ratio of cells with positive IHC staining for CDK1 pT14 (panel C) and γH2AX (panel D) in tumor slides shown in panel B. **E and F.** Tumor measurement following RP-6306 treatment in nude mice with transplanted PDX BCX070 (LMW-E high, panel E) or PDX XC5172013 (LMW-E low, panel F). **G.** Representative IHC images for CDK1 pT14, γH2AX and Ki67 in end-of-treatment tumors collected in panel E and F. **H - M.** Quantitation of the ratio of cells with positive IHC staining for CDK1 pT14 (panel H and I), γH2AX (panel J and K), and Ki67 (panel L and M) in tumor slides shown in panel G.

Conclusions

Our results reveal that LMW-E and PKMYT1-mediated-CDK1 pT14 expression are associated with worse clinical outcomes in TNBC patients who were treated with neoadjuvant chemotherapy. In TNBC cell lines, inhibiting PKMYT1 with first-in-class small molecule inhibitor RP-6306 reduced cell viability in an LMW-E dependent manner. Treatment of LMW-E high tumor cells with RP-6306 resulted in accumulation of sub-G1 and polyploid cell population, caused apoptosis and accumulation of chromosomal breakage with increased DNA damage. In TNBC *in vivo* models such as MMTV-LMW-E driven mammary tumors, and LMW-E high PDX models, RP-6306 selectively induced DNA damage and reduced tumor growth. ***Collectively, our results show that overexpression of LMW-E and CDK1-pT14 in TNBC can be used to stratify patients whose tumors are likely to respond to RP-6306.***

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