Final Report

Roles of nc886 Suppression in Pro-Inflammatory Signals of Cholangiocarcinoma

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Roles of nc886 Suppression in Pro-Inflammatory Signals of Cholangiocarcinoma

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Abstract

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Abstract:

Protein Kinase R (PKR), a pro-apoptotic protein in innate immunity and cancer, induces apoptosis when treated with doxorubicin, a widely used chemotherapeutic drug in malignancies. However, the doxorubicin-sensing mechanism and therapeutic significance have remained undefined. Here we have discovered a novel mechanism that involves nc886, a cellular non-coding RNA (ncRNA). nc886 is transcribed by RNA polymerase III (Pol III), binds to PKR, and prevents it from aberrant activation. Doxorubicin evicts Pol III from DNA and thereby shuts down nc886 transcription, leading to its acute decrease enabling PKR activation and ultimately apoptosis. Our study has identified nc886 as a molecular signal for PKR to sense doxorubicin and has future clinical potential by providing a selective treatment regimen depending upon the nc886/PKR status of cancer cells.

Keywords : nc886, PKR, Doxorubicin, Cancer, Chemotherapy

Abstract

Protein Kinase R (PKR), a pro-apoptotic protein in innate immunity and cancer, induces apoptosis when treated with doxorubicin, a widely used chemotherapeutic drug in malignancies. However, the doxorubicin-sensing mechanism and therapeutic significance have remained undefined. Here we have discovered a novel mechanism that involves nc886, a cellular non-coding RNA (ncRNA). nc886 is transcribed by RNA polymerase III (Pol III), binds to PKR, and prevents it from aberrant activation. Doxorubicin evicts Pol III from DNA and thereby shuts down nc886 transcription, leading to its acute decrease enabling PKR activation and ultimately apoptosis. Our study has identified nc886 as a molecular signal for PKR to sense doxorubicin and has future clinical potential by providing a selective treatment regimen depending upon the nc886/PKR status of cancer cells.

Executive summary

Doxorubicin, an anticancer drug, has been widely used for cancer treatment for nearly a half of century but the precise mechanism underlying its cytotoxicity remains to be identified. There are numerous reports that Protein Kinase R (PKR), an apoptotic protein known to response to different stimuli, is activated upon treatment with doxorubicin and subsequently promotes apoptosis. Nonetheless, the mechanism of how doxorubicin activates PKR is completely unknown. We have identified a novel regulator that binds to PKR and suppresses its activation. It is a non-coding RNA 886 (nc886) whose transcription is mediated by RNA polymerase III (Pol lll). The inhibition of Pol III activity by doxorubicin has also been documented.

Collectively nc886 is an interesting candidate to be a signaling molecule that links PKR and doxorubicin to therapeutic effect of cancer drug. Therefore, in this report, we aimed to examine how nc886 mediates PKR activation and is regulated by Pol lll upon doxorubicin treatment. We perused these aims by performing several experiments. First, we determined nc886 expression upon doxorubicin treatment in a variety of cancer cell lines by northern blot hybridization. Second, we tested the PKR's contribution in doxorubicin induced apoptosis in PKR knock out cell lines generated by the CRISPR-Cas9 technique. Next, we evaluated the nc886's contribution to doxorubicin mediated cytotoxicity by nc886 overexpression using in vitro transcribes. Finally, we examined the mechanism of nc886 suppression by doxorubicin by performing Chromatin Immunoprecipitation Assay (ChIP).

Result

1. nc886 is decreased upon doxorubicin treatment.

nc886 was decreased by doxorubicin in a variety of other cell lines: an immortalized thyroid cell line Nthy-ori 3-1 [PKR wild type (wt)] and its derivative PKR knockout cell line (PKR KO), a immortalized cholangiocyte cell line MMNK1, a cholangiocarcinoma cell line M214, and a breast cancer cell line MDA-MB-231. Our consistent data across cell lines of diverse tissues origins (lung, thyroid, and bile duct) suggest that the suppression of nc886 expression by doxorubicin is a ubiquitous phenomenon.

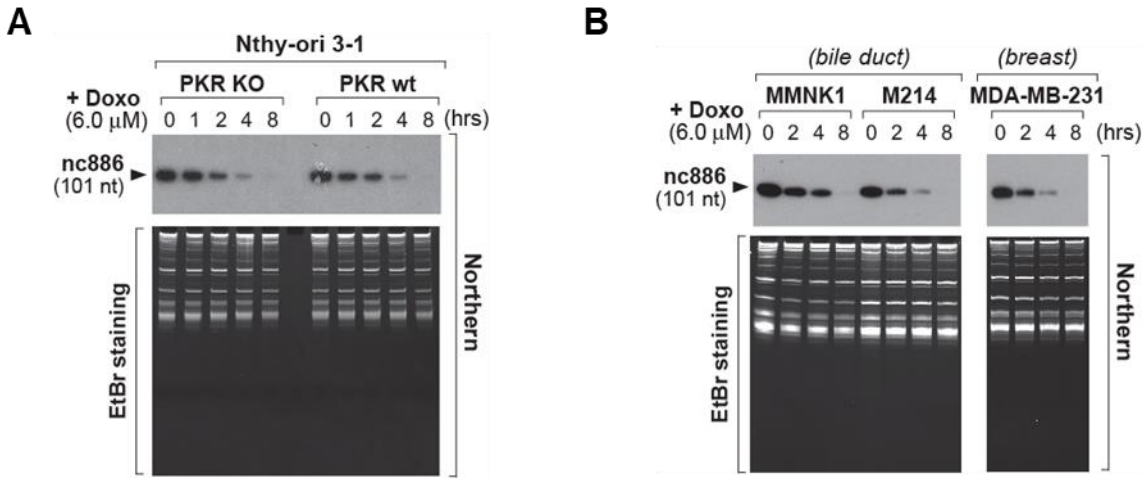

Figure 1. A-B. Northern blot of nc886 and EtBr staining for a loading control. Doxorubicin treatment conditions are displayed in figure captions.

2. PKR contributes to doxorubicin-induced apoptosis.

Doxorubicin has been shown to provoke the PKR pathway and ultimately apoptosis. In the case of human cells, the experimental evidence for PKR's contribution to this apoptotic pathway has been mostly based on knockdown (KD) experiments by using small interfering (or hairpin) RNA (siRNA or shRNA). This role of PKR has been corroborated by our experiments with PKR KO cells that were generated from the original Nthy-ori 3-1 cells (PKR wt) by the CRISPR-Cas technique. The PKR status was assured by immunoblot examination of phospho-PKR and total PKR (Fig 2A).

Doxorubicin treatment induced apoptotic marker proteins, such as caspase-3 and cleaved Poly (ADP-ribose) polymerase (PARP) in PKR wt cells, but this induction was significantly attenuated in PKR KO cells (Fig 2A).

The comparable induction of p53 and Chk2 (as indicated by their phospho-forms in Fig 2A) in PKR wt and KO cells ascertained that doxorubicin was equally efficient in these two cell lines and that our apoptosis data could not be attributed to a difference in DNA damage responses. We also performed MTT cell proliferation assays upon treatment with titrating amounts of doxorubicin (Fig 2B). PKR KO cells were more resistant to doxorubicin than PKR wt (IC50 value = 22.83 vs 6.49 μM respectively.

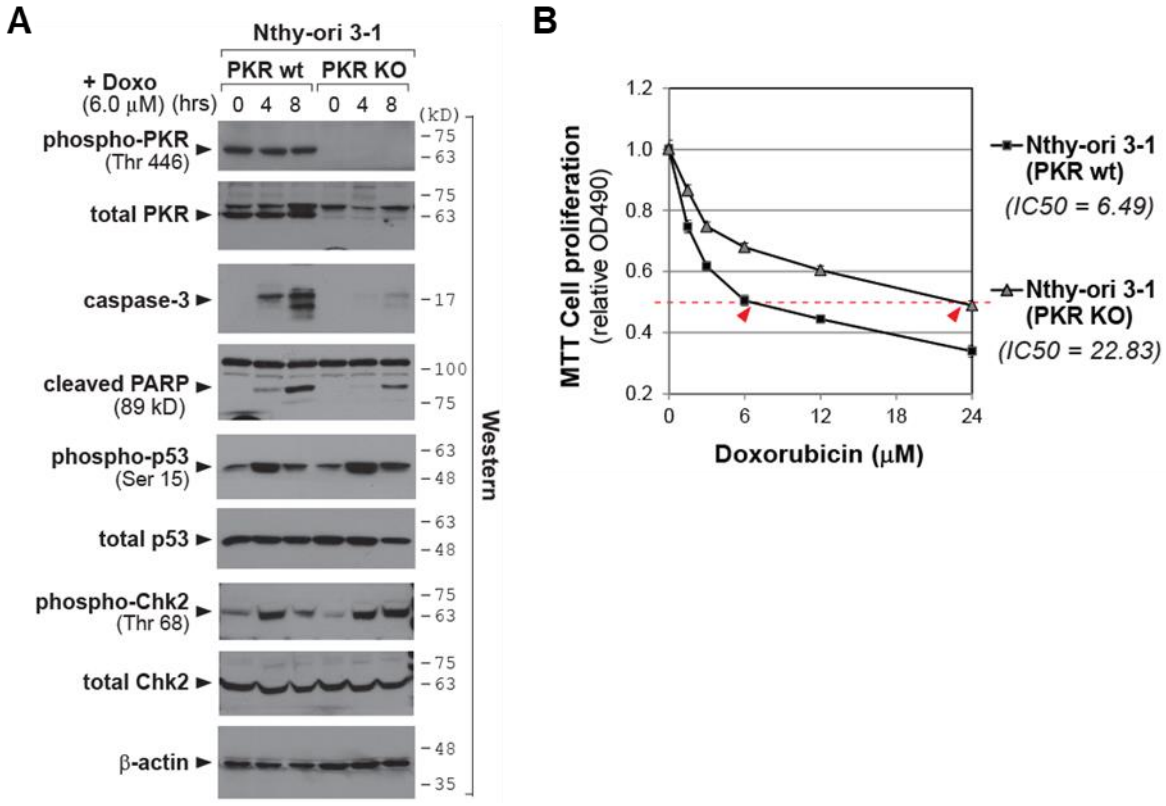

Figure 2. A. Western blot of indicated proteins after doxorubicin treatment. Molecular size markers (in kilo-Dalton) are indicated on the right.

B. MTT cell proliferation assays. Doxorubicin was treated for 24 hr. The IC50 values are indicated below each cell line. Each value is an average of triplicate samples and the standard deviation is not shown for simplicity of the plot.

3. nc886's role in the PKR-mediated cytotoxic effect upon doxorubicin treatment

We wanted to discern nc886's contribution and interrogated whether doxorubicin-mediated cytotoxicity was mitigated by ectopic expression of nc886. Our strategy for nc886 expression in the presence of doxorubicin was to deliver it in a form of an in vitro transcript. Doxorubicin-induced apoptosis was attenuated when the nc886 transcript was transfected into HCT116 and Nthy-ori 3-1 (PKR wt) cells, whereas such attenuation was not seen in Nthy-ori 3-1 (PKR KO) cells (Fig 4A-B). The role of nc886 in the PKR pathway was further confirmed by testing two versions of nc886, one with the wt sequence and the other with a mutation at nt 46-56 where PKR made contact with nc886 ("mut_46-56") (Fig 3C). This mutant is deficient in PKR binding. Doxorubicin-induced apoptosis, as indicated by caspase-3 induction, was mitigated by wt nc886 but not by mut_46-56 (Fig 3C). All of our data unequivocally demonstrated that PKR activation by nc886 suppression plays a significant role in the cellular response to doxorubicin.

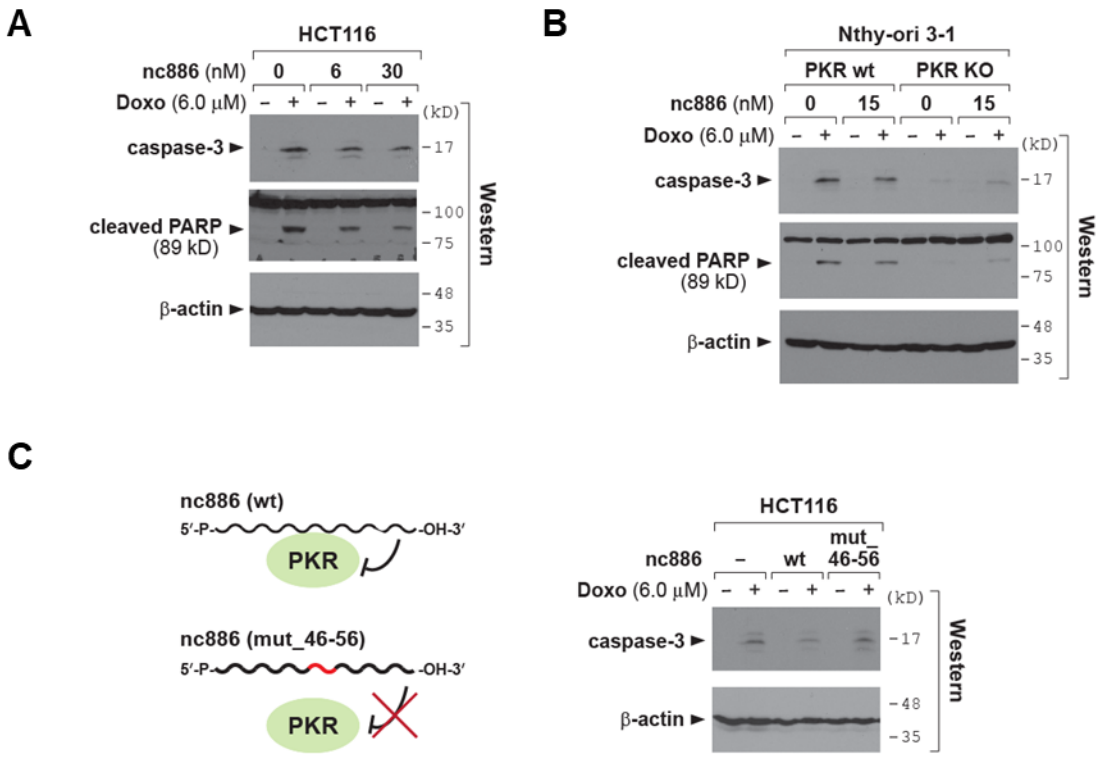

Figure 3. A-B. Transfection of the nc886 RNA (in vitro transcript) combined with doxorubicin treatment. Transfection and treatment were done simultaneously before harvesting cells at 12 hr (HCT116) or 6 hr (Nthy-ori 3-1) for Western blot. Each transfection was adjusted to contain the same total μg amount of RNA by adding yeast tRNA to 2 μg in the transfection mixture for a 6-cm dish.

C. The same experiment as panel B-C, except that a mutant nc886 was used. The feature of the mutant nc886 as compared to wt is briefly illustrated in the left panel, with the mutated portion highlighted in red. Mutant nc886 was transfected at 15 nM. The same concentration of wt nc886 and the same μg of yeast tRNA (designated "-") were transfected as a positive and negative control respectively.

4. The eviction of Pol III and inhibition of transcription by doxorubicin

Because doxorubicin is known to evict histones, we surmised that these two drugs would also evict Pol III. Our chromatin immunoprecipitation (ChIP) with a Pol III subunit (POLR3A), showed that POLR3A was specifically bound to Pol III loci, nc886 and vtRNA1-1, but not at a GAPDH locus which is a Pol II gene and that this association was significantly decreased by doxorubicin (Fig 4).

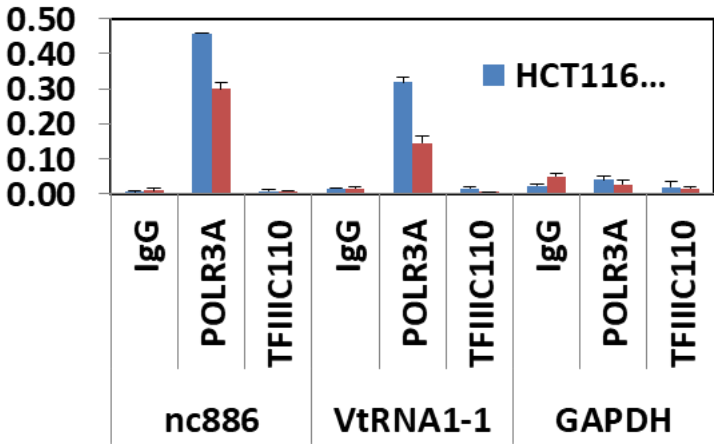

Figure 4. qPCR of indicated genomic regions bound to POLR3A, after treatment of 6 μM doxorubicin for 2 hr. $2^{-\Delta Ct}$ values from POLR3A and IgG ChIP DNA were normalized to input DNA to calculate fold-enrichment (y-axis). The measurement was in triplicates.

Conclusion and Discussion

In this study we have established that nc886/PKR signaling is another important pathway in doxorubicin chemotherapy. Despite PKR's apoptotic role therein, the mechanism as to how PKR senses doxorubicin has remained unknown. We have answered this conundrum by identifying nc886 as the molecular signal. We expect our data to be applicable to optimizing the therapeutic regimen for doxorubicin treatment according to nc886/PKR status. Due to PKR's pro-apoptotic function, PKR is supposed to be suppressed in malignancies and it has been shown so in a number of literatures. In line with this, nc886 expression is ordinarily elevated in immortalized or transformed cells as compared to normal cells, because global Pol III transcription increases during tumorigenesis. Therefore, doxorubicin might provoke the nc886/PKR pathway more potently in neoplastic cells (with high nc886 but low PKR) than in normal cells. There is another subset of cancer cells in which nc886 is epigenetically silenced. In this subset, we consider the above doxorubicin regimen to be inappropriate, but would suggest that PKR inhibitory drugs be included in the regimen. That is because doxorubicin mainly targets dividing tumor cells, but could harm normal cells possessing PKR-mediated innate immunity via the nc886/PKR pathway. In summary, our finding could be harnessed to ameliorate doxorubicin treatment for an optimal therapeutic window and minimal adverse effects.

Output

Kunkeaw et. al. Doxorubicin induces Protein Kinase R-mediated apoptosis via suppressing the non-coding RNA nc886. Elife. (Manuscript in preparation)