Published in final edited form as:

Oncogene. 2013 August 8; 32(32): . doi:10.1038/onc.2012.382.

Cell death/proliferation roles for nc886, a non-coding RNA, in the Protein Kinase R pathway in cholangiocarcinoma

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Abstract

We have recently identified nc886 (pre-miR-886 or vtRNA2-1) as a novel type of non-coding RNA that inhibits activation of PKR (Protein Kinase RNA-activated). PKR's pro-apoptotic role through eIF2 α phosphorylation is well established in the host defense against viral infection. Paradoxically, some cancer patients have elevated PKR activity; however, its cause and consequence are not understood. Initially we evaluated the expression of nc886, PKR and eIF2 α in non-malignant cholangiocyte and cholangiocarcinoma (CCA) cells. nc886 is repressed in CCA cells and this repression is the cause of PKR's activation therein. nc886 alone is necessary and sufficient for suppression of PKR via direct physical interaction. Consistently, artificial suppression of nc886 in cholangiocyte cells activates the canonical PKR/eIF2 α cell death pathway, suggesting a potential significance of the nc886 suppression and the consequent PKR activation in eliminating pre-malignant cells during tumorigenesis. In comparison, active PKR in CCA cells does not induce phospho-eIF2 α nor apoptosis, but promotes the pro-survival NF- κ B pathway. Thus, PKR plays a dual life or death role during tumorigenesis. Similarly to the CCA cell lines, nc886 tends to be decreased but PKR tends to be activated in our clinical samples from CCA patients. Collectively from our data, we propose a tumor surveillance model for nc886's role in the PKR pathway during tumorigenesis.

Keywords

nc886; cholangiocarcinoma; PKR; eIF2 α ; NF- κ B

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

We have recently characterized a novel type of non-coding RNA, nc886 (pre-miR-886 or vtRNA2-1) (1). As its aliases indicate, it was originally known as a precursor microRNA (2) or as an RNA component of the vault complex (3, 4). However, we have shown that it is neither a precursor microRNA nor a vtRNA (1); so we have renamed it nc886 (*non-coding RNA-886*) and this term will be used throughout this paper and in the future. A brief summary of nc886 is: 1) its expression is ubiquitous in non-malignant human tissues but suppressed in a wide range of cancer cells, and 2) its artificial suppression leads to the activation of PKR (*Protein Kinase RNA-activated*) and impaired cell proliferation (1). Recently, nc886's significance in the biology and the prognosis of acute myeloid leukemia has been reported (5).

PKR is an interferon-inducible and double-stranded RNA (dsRNA)-dependent kinase, whose role has been well established in the host defense against viral infection. In the canonical PKR pathway [reviewed in (6)], virus-derived dsRNA binds to PKR and mediates its autophosphorylation. Phosphorylated PKR (P-PKR) is an active kinase that phosphorylates eIF2 α (*eukaryotic initiation factor 2 α subunit*) at Ser51. Phosphorylated eIF2 α (P-eIF2 α) blocks global protein synthesis by preventing its efficient recycling by the guanine nucleotide exchange factor eIF2B (*eukaryotic translation initiation factor 2B*), and subsequently leads to cell death (7-9). PKR also has the dual role of activating the NF- κ B pathway which promotes cell proliferation [reviewed in (10)].

In addition to its established role in the interferon response, PKR is involved in many pathways and exerts various functions on cell growth independently of viral infection [reviewed in (11)]. Thus, not surprisingly, PKR is implicated in tumorigenesis. However, PKR's exact role in cancer biology remains controversial. Initially, PKR was thought to be a tumor suppressor. Early studies indicated that malignant transformation was induced by dominant-negative mutants of PKR (and thus its loss of function). This corroborated PKR's pro-apoptotic role. It has been also reported that PKR was suppressed or inactivated in some malignancies (12-16). However, several observations have challenged the proposed role of PKR as a tumor suppressor. In some cancers, PKR and its downstream eIF2 α are induced in expression and activity (1, 5, 17-29). In addition, NF- κ B, a pro-proliferative factor which is the downstream of PKR, is also activated in many cancers [reviewed in (10)].

What is the biological significance of activated PKR in cancer? Our data about nc886, together with the previous knowledge of PKR, led us to propose a tumor surveillance model for the nc886/PKR role in cancer biology. In this model, decrease of nc886 during early stages of tumorigenesis activates the classical PKR/eIF2 α pathway which leads to cell death and eliminate most precancerous and/or cancerous cells. Cancer cells that have survived must have developed bypass mechanisms to proliferate in the presence of elevated P-PKR and P-eIF2 α . Once cancer cells have overcome the PKR/eIF2 α cell death pathway, P-PKR could play the pro-proliferative role necessary for further tumor progression through its downstream NF- κ B pathway.

In this paper, we have investigated our tumor surveillance model in cholangiocarcinoma (CCA), a cancer from the hepatobiliary epithelium. This study is the first step to elucidate the roles of nc886/PKR in cell death/proliferation during tumorigenesis.

Results

Expression of nc886, PKR, and eIF2 α in cholangiocyte and CCA cells

We measured nc886 in a battery of bile duct cells (Table 1). Like other non-malignant human cells in our previous studies (1), nc886 was expressed in immortalized but not malignant cholangiocyte MMNK1 cells (Fig 1). nc886 was also expressed in three CCA cells (M156, M214, and M055), whereas it was not expressed in M213, its derivative lines M213L0H and M213L5H, and M139 cells. For comparison, all of the bile duct cells constitutively expressed vtRNA1-1, a canonical vtRNA (Fig 1).

While PKR was constitutively expressed in cholangiocyte and CCA cells (total PKR in Fig 1), it was activated in all the CCA cells except for M156, as indicated by P-PKR Western blot (Fig 1). Similarly to P-PKR, P-eIF2 α was elevated in all the CCA cell lines, but again not in the non-malignant MMNK1 (Fig 1). In our experiments in Fig 1, it should be noted that all assays were performed in a normally growing condition without exposure to dsRNA, a natural activator of PKR that leads to apoptosis.

In general, CCA cells tended to have decreased nc886 but elevated P-PKR/P-eIF2 α relative to non-malignant MMNK1 cells (Fig 1 and Table 1). Certainly, there were exceptional cell lines; nc886 was not silenced in all the CCA cells, and nc886 was co-expressed with P-PKR in M214 and M055 cells. This is explained by the genetic diversity of CCA cells. PKR has been known to be regulated by a number of intra-/extra-cellular factors besides dsRNA [reviewed in (6)]. For example, natural PKR regulators such as PACT or p58IPK may have contributed to the activation of PKR in M214 and M055 cells. We speculate that nc886 is also under complex regulation, as a number of transcription factors are predicted to bind to the vicinity of the nc886 locus.

Taken together with our previous result that suppression of nc886 activates PKR to P-PKR (1), the anti-correlation between nc886 and P-PKR in a battery of cholangiocyte/CCA cells (Fig 1) was intriguing and raised these questions: 1) is the expression of nc886, P-PKR, and P-eIF2 α related causally during tumorigenesis? and 2) how do CCA cells overcome P-PKR/P-eIF2 α 's apoptotic effect and proliferate? We addressed these issues in the following experiments. As we used several cell lines with different expression of nc886, P-PKR, and P-eIF2 α , we designated their expression by +/- signs in a parenthesis, for easy reading. For example, MMNK1 cells are (nc886+, P-PKR-, P-eIF2 α -; see also Table 1).

Causal relationship between nc886 and P-PKR

A number of papers have reported PKR activation in malignancies; however, most of them did not elucidate a mechanism for that activation. In one report (18), a PKR inhibitor protein p58IPK (30) was suggested to be the cause, because p58IPK was decreased in breast cancer cells whereas PKR was activated. However, this observation was not reproducible in other papers, and thus p58IPK's universal involvement in PKR regulation seemed unlikely (17, 20). In this study we found that the expression of p58IPK was consistent among the cell lines (Fig 2A), and therefore ruled out its role in PKR regulation in CCA.

Based on our data in Fig 1 and our previous study (1), we hypothesized that suppression of nc886 activated PKR in CCA cells. To investigate the nc886/PKR relationship, we suppressed nc886 by transfecting antisense oligonucleotides (anti-oligos) into MMNK1, M156, and M214 cells (all nc886+ cells) and then measured P-PKR. Compared to an anti-oligo against vtRNA1-1 ("anti-vt21_2") as a negative control, anti-oligos against nc886 ("anti886s") successfully negated nc886 and induced P-PKR in all the three cell lines examined (Fig 2B-D). Interestingly, there were two distinct mechanisms for anti-oligos to induce P-PKR. In the case of non-malignant MMNK1 cells, the anti-oligos depleted nc886's

intracellular level (Northern blot in Fig 2B). In M156 and M214 CCA cells, the anti-oligo interfered with nc886/PKR interaction (pull-down assay in Fig 2C).

Our cellular data were reinforced by *in vitro* experiments. Addition of the anti-oligo against nc886 in MMNK1 lysate (nc886+) increased PKR activity, as measured by *in vitro* kinase assays (Fig 2E). Conversely, addition of *in vitro* transcribed synthetic nc886 in M213 lysate (nc886-) inhibited PKR activity (Fig 2F). We also showed their direct physical interaction by electrophoretic mobility shift assays (EMSA) with synthetic nc886 and purified PKR from *E. coli* (Fig 2G).

Collectively, our data demonstrated that nc886 alone is necessary and sufficient for suppression of PKR through direct physical interaction. nc886 keeps PKR repressed in MMNK1 cells, and PKR is released from that repression when nc886 is suppressed in CCA cells. However, nc886 levels could not explain P-PKR in all the CCA cells, for example, in M214 and M055 (nc886+, P-PKR+). As mentioned earlier, their intrinsic level of P-PKR could have been activated by a factor other than nc886 or p58IPK, or in these malignancies there seems to be a higher threshold level of nc886 necessary for suppression of P-PKR.

The canonical PKR/ eIF2 α pathway operates in MMNK1 cells but not in CCA cells

One pre-requisite for our tumor surveillance model is that the canonical PKR/eIF2 α pathway operates normally and leads to apoptosis in most cells, because the suppression of nc886 would eliminate such cells through this pathway. We attempted to recapitulate such a situation in non-malignant MMNK1 cells (nc886+, P-PKR-, P-eIF2 α -). Upon nc886 depletion, P-PKR phosphorylated eIF2 α , decreased global protein synthesis and inhibited cell proliferation (Fig 3A-B).

P-PKR is known to induce apoptosis mainly through two pathways involving FADD/ caspase-8 and APAF/ caspase-9, both of which merge onto caspase-3 [reviewed in (6)]. Consistently, we detected the active form of cleaved caspase-3 and consequent cleavage of PARP (poly(ADP-ribose) polymerase) upon nc886 knockdown in MMNK1 cells (lane 1-2 in Fig 3C). In contrast, neither caspase-3 nor PARP cleavage was seen in M156 and M214 CCA cells (lane 3-6 in Fig 3C), although PKR was indeed activated by nc886 depletion (Fig 2D). Hence, there were two distinct consequences of P-PKR activity between MMNK1 cells and CCA cells. It is worth noting that M156 and M214 CCA cells were both P-eIF2 α + (Fig 1).

To interrogate which step of the PKR pathway is abrogated in these two cell lines, we measured P-eIF2 α and global protein synthesis upon nc886 knockdown (Fig 3D). In the case of M156 cells (nc886+, P-PKR-, P-eIF2 α +), P-eIF2 α was induced but global protein synthesis was not significantly decreased. In the other case of M214 cells (nc886+, P-PKR+, P-eIF2 α +), the induction of P-eIF2 α was not seen and consistently global protein synthesis was unaffected.

So far, we have shown that the canonical PKR/eIF2 α pathway operated normally in non-malignant MMNK1 cells, but not in CCA cells. Phosphorylation of eIF2 α (M214) or inhibition of global protein synthesis (M156) malfunctioned so that the two CCA cells escaped from apoptosis upon nc886 suppression.

The PKR pathway upon introduction of dsRNA

Next, we expanded our investigation to CCA cells lacking nc886. To activate the PKR/ eIF2 α pathway, we transfected a dsRNA mimic Poly(I:C) into M139 cells (nc886-, P-PKR+, P-eIF2 α +). For comparison, we included two cell lines, MMNK1 (nc886+, P-PKR-, P-eIF2 α -) and M214 (nc886+, P-PKR+, P-eIF2 α +) in these experiments.

Poly(I:C) activated PKR in all the cell lines tested (Fig 4A). The intact PKR/eIF2 α pathway was again confirmed in non-malignant MMNK1 cells in which Poly(I:C) inhibited global protein synthesis via P-eIF2 α (Fig 4A). In contrast, P-PKR did not further phosphorylate eIF2 α nor decrease global protein synthesis in M214 and M139 cell lines (Fig 4A). In M214 cells, Poly(I:C) treatment and nc886 knockdown yielded the same result (compare Fig 4A and 3D). So, P-PKR failed to phosphorylate eIF2 α in these two cells.

This raised a question as to whether P-PKR also failed to activate its other downstream events. As eIF2 α and NF- κ B are two representative downstream branches in the PKR pathway, we examined the NF- κ B pathway (Fig 4B). Poly(I:C) treatment activated the NF- κ B pathway in all the three CCA cell lines tested, including M214 and M139 where P-PKR failed to phosphorylate eIF2 α . This NF- κ B activation was PKR-dependent, as it was abrogated by 2-aminopurine (2-AP), a PKR inhibitor (Fig 4B). Thus, M214 and M139 CCA cells selectively blocked the eIF2 α branch, but not the NF- κ B branch in the PKR pathway.

The NF- κ B pathway in CCA cells

As the activation of NF- κ B by P-PKR was operative in the presence of dsRNA, we questioned if the intrinsic NF- κ B activity was elevated in CCA cells (P-PKR+) relative to MMNK1 cells (P-PKR-). First, we wanted to ensure that PKR activity was indeed higher in CCA cells than in MMNK1 cells by employing *in vitro* kinase assays. Consistent with our earlier P-PKR Western data (Fig 1), M214 and M213 cells (P-PKR+) had a robust PKR kinase activity, compared to the very low activity of the MMNK1 cell line (P-PKR-) (Fig 5A). Accordingly, the NF- κ B activity of these two CCA cells was higher than that of MMNK1, as assessed by luciferase assays measuring the NF- κ B-responsive promoter activity (Fig 5B) and by qRT-PCR measurement of NF- κ B target mRNAs (Fig 5C).

The dependence of NF- κ B activity on P-PKR (thus PKR's kinase activity) was reinforced by knockdown experiments. In M156 and M214 (both nc886+), nc886 knockdown resulted in PKR activation in our earlier data (Fig 2D). Consistently, NF- κ B was activated, as shown by qRT-PCR of NF- κ B target genes (Fig 5D). This activation was significantly decreased by simultaneous knockdown of PKR by siRNA, demonstrating the requirement of PKR for the NF- κ B activation by nc886 depletion.

Finally, we examined if the elevated PKR/NF- κ B activity played a role in the proliferation of CCA cells. For this, we treated cells with caffeic acid phenethyl ester (CAPE), a chemical inhibitor for NF- κ B, and measured cell proliferation. CAPE treatment suppressed proliferation of M214 and M213 (both P-PKR+) CCA cells (Fig 5E), demonstrating NF- κ B's pro-proliferative role therein. A similar effect of CAPE on CCA growth has been reported previously (31). In agreement, inhibition of PKR activity by 2-AP treatment also attenuated the proliferation of these CCA cells (Fig 5F).

The elevated P-eIF2 α in CCA cells

The intrinsic expression of P-eIF2 α in CCA cells was still intriguing but not addressed yet. An important obvious question was what caused eIF2 α to be phosphorylated. The primary candidate was certainly P-PKR, an eIF2 α kinase, because its expression was positively correlated with P-eIF2 α (Fig 1). However, this was questionable, because eIF2 α was not phosphorylated by P-PKR in CCA cells upon nc886 knockdown or dsRNA treatment (Fig 3-4). To test this, we suppressed PKR in M214 and M213 cell lines (both P-PKR+, P-eIF2 α +). siRNAs against PKR successfully decreased total PKR and P-PKR, but not P-eIF2 α (Fig 6A). Thus, it is unlikely that P-eIF2 α was induced by P-PKR in these cell lines.

Another candidate was MetAP2(p67), as it is known to bind eIF2 and protect eIF2 α from phosphorylation by P-PKR [reviewed in (32)]. We found its expression to be higher in CCA cells than in MMNK1 (Fig 6B). These data were against our scenario that eIF2 α would be released from its protection and hyper-phosphorylated when MetAP2(p67) decreased in CCA. Our data suggested that P-eIF2 α was induced in CCA cells by some factors other than P-PKR or MetAP2(p67).

An equally important question was how CCA cells could proliferate in the presence of P-eIF2 α . Obviously, their protein translation normally occurred in spite of P-eIF2 α 's intrinsic expression (Fig 1), as well as its induction by nc886 knockdown (M156 in Fig 3D). P-eIF2 α 's mechanism for inhibition of protein translation is to bind to eIF2B with an excessively high affinity and consequently deplete productive eIF2B, an essential factor for protein translation (33). Accordingly, overexpression of eIF2B has been shown to negate P-eIF2 α 's inhibitory effect on protein translation (34, 35). In addition, high expression of eIF2B in malignancies has been reported (18, 36). We measured eIF2B- ϵ , the catalytic subunit of the penta-peptide eIF2B complex (37), and found it to be elevated in CCA cell lines relative to MMNK1 (Fig 6B). Thus it is likely that compensating high levels of eIF2B was one mechanism by which CCA cells overcame P-eIF2 α .

nc886 and P-PKR expression in clinical samples

Thus far, we have shown the significance of nc886 in the PKR pathway in the cholangiocyte/CCA cell culture. To corroborate our *in vitro* findings, we measured nc886 in clinical specimens from CCA patients. Among 16 pairs of CCA tumors and adjacent normal tissues, nc886 decreased in tumor in eight cases, increased in four cases, and did not change significantly in four cases (Fig 7A). This pattern was very similar to that observed in the seven CCA cell lines (Fig 1). Compared to MMNK1, nc886 decreased in four CCA cell lines and increased in one CCA cell line.

Next, we measured P-PKR in another set of eight CCA samples with matched normal liver tissue samples by immunohistochemistry (IHC). P-PKR was strongly expressed in CCA but not at all in surrounding fibrous tissues (Fig 7B). In six of eight patients, the P-PKR expression in CCA was stronger than in matched bile ducts or liver tissues (Fig 7B-C). Thus, we have provided evidence here that the nc886/P-PKR expression in CCA patients was similarly altered to that observed in the CCA cell lines (Fig 1). Although accumulation of more clinical data will be needed to assess nc886/P-PKR's *in vivo* significance more accurately, we suggest that the nc886/PKR tumor surveillance mechanism could operate *in vivo*.

Discussion

In this report, we substantiated that a non-coding RNA, nc886, represses PKR. We have provided clear evidence that nc886 directly binds to PKR and inhibits its phosphorylation and kinase activity (Fig 2). Their molecular interaction is being characterized in detail (SHJ and YSL; manuscript in preparation). The high P-PKR (thus elevated PKR activity) in CCA cells, albeit not all of them, is attributed to suppression of nc886 therein. This causal relationship is probably true also in breast cancer cells, as we found an anti-correlation between nc886 and P-PKR by comparing published data (18) to our data (1).

Our data presented here in CCA support our tumor surveillance model, which is described in the Introduction and illustrated in Fig 8. In non-malignant MMNK1 cells, artificial suppression of nc886 led to apoptosis through the classical PKR cell death pathway via P-eIF2 α (Fig 3). We presume that the canonical PKR/eIF2 α pathway is intact in most pre-

malignant cells and eliminates these cells when nc886 is suppressed during certain stages of tumorigenesis, similarly to MMNK1 cells.

However, cancer is a complex genetic disease involving an accumulation of mutations. By chance, these mutations could confer upon cells mechanisms to subvert the PKR/eIF2 α cell death pathway. Such malignant cells would survive a high expression of P-PKR and/or P-eIF2 α , as exemplified by the CCA cells in this study. Such bypass mechanisms seem to be diverse. Herein we have identified two distinct survival mechanisms: 1) the failure of eIF2 α phosphorylation by P-PKR (M214 and M139) and 2) sustained global protein synthesis despite P-eIF2 α (M156). At the molecular level, there may be myriads of such mechanisms which await further study.

Regarding the modified PKR pathways that CCA cells have developed, several points should be discussed. First, the P-eIF2 α branch ceased to function, but P-PKR and its NF- κ B branch remained intact in all the CCA cells tested here. Although CCA cells were P-PKR+, PPKR was further induced by nc886 depletion (Fig 2) or introduction of dsRNA (Fig 4), and the P-PKR activated NF- κ B (Fig 5). Second, the elevated intrinsic expression of P-eIF2 α in CCA cells was independent of P-PKR (Fig 6A). This was surprising, given their established relationship. However, PKR is not the only kinase for eIF2 α . Besides PKR, there are at least three kinases that phosphorylate eIF2 α [reviewed in (38)]. P-eIF2 α is also regulated by its de-phosphorylation (39, 40). Currently, we do not know what causes the intrinsic expression of P-eIF2 α . In any case, we have shown that one mechanism for CCA cells to tolerate P-eIF2 α was overexpression of eIF2B (Fig 6B).

Collectively from our data obtained from CCA cells, we propose an updated tumor surveillance model. First, cells develop a mechanism (for example, eIF2B overexpression) by which they survive in spite of the classical P-eIF2 α effects. Perhaps, this process is stochastic by random mutations. In any case, these cells are able to survive, even if nc886 is suppressed and thereby PKR is activated during tumorigenesis. Once these cells bypass the nc886/PKR/eIF2 α action, they take advantage of P-PKR's oncogenic role by virtue of its ability to activate NF- κ B. Consistently, we and others have shown that NF- κ B activity is higher in CCA and required for CCA cell proliferation [Fig 5 and (31)].

Given that nc886 (1) and PKR [reviewed in (6)] are expressed ubiquitously, we expect that the tumor surveillance mechanism operates in a wide range of cancers besides CCA. Additionally, the suppression of nc886 may be used for therapeutic purposes or as prognosis of cancer progression. We will expand our study from CCA to other types of cancer, such as breast, lung, and head-and-neck cancers (1). Obviously, there are many unanswered questions. For example, what triggers the suppression of nc886, the first critical event in the nc886-PKR pathway? What is the molecular mechanism for the modified P-eIF2 α branch? Most importantly, an animal model system needs to be developed in order to evaluate the *in vivo* contribution of the nc886/PKR tumor surveillance mechanism to cancer incident rates.

Materials and Methods

Cell lines, antibodies, and other reagents

MMNK1 cholangiocyte and CCA cells (summarized Table 1) were a kind gift from Dr. Sopot Wongkham (Khon Khan University, Thailand). Antibodies were obtained as follows: p58IPK, caspase-3 (cleaved), AIF, PARP (cleaved), eIF2B- ϵ from Cell Signaling Technology (Danvers, MA); MetAP2(p67) from Invitrogen (Carlsbad, CA). All other antibodies were described in (1). Basic chemicals and reagents, including 2-AP and Fumagillin, were purchased from Sigma-Aldrich (St. Louis, MO). CAPE was purchased from Tocris Bioscience (Minneapolis, MN).

Plasmid DNAs, RNAs, and transfection

pNF- κ B-Luc (Stratagene, La Jolla, CA), pRL-SV40 (Promega, Madison, WI), and pcDNA3.1-Zeo(+)-Pp (1) were used for luciferase assays. siRNAs against PKR were Stealth RNAi™ siRNA (Invitrogen) whose sequences are available upon request. Yeast tRNA was purchased from Applied Biosystems/Ambion (Carlsbad, CA). Anti-oligos (against nc886 and vtRNA1-1), synthetic nc886, and Poly(I:C)-LMW (0.2-1 kb) were purchased or prepared as described in (1). siRNA and anti-oligos were transfected with Lipofectamine™ RNAiMAX reagent (Invitrogen). Poly(I:C), yeast tRNA, and plasmid DNAs were transfected with Lipofectamine™ 2000 reagent (Invitrogen),

Assays (measurement/analysis of RNA, luciferase assays, and cell proliferation assays)

All these assays were performed as previously described in (1), except that BrdU incorporation assays were performed by using Cell Proliferation ELISA BrdU (Roche Applied Science, Indianapolis, IN).

In vitro kinase assay or measurement of nc886 following PKR immunoprecipitation

PKR immunoprecipitation was performed as described in (41), except that all the buffers contained 80 units/ml of RNase inhibitor (New England Biolabs, Ipswich, MA) and 1 mM Ribonucleoside Vanadyl Complex (New England Biolabs). Washed immunoprecipitates (bound to beads) were subjected to different further manipulations per experimental purposes. To test nc886 association with PKR (Fig 2C experiments), the beads were resuspended with Trizol reagents (Invitrogen) for RNA isolation. During RNA isolation, 5 μ g of yeast tRNA was added as a carrier for isopropanol precipitation. To measure *in vitro* kinase activity of PKR (Fig 2E-F and Fig 5A experiments), the beads were resuspended in 20 μ l of kinase buffer (41) containing 2 μ Ci of [γ -³²P]-ATP and incubated at 30°C for 20 min. The reaction was quenched by adding 2X SDS-PAGE loading buffer and resolved on a 10% SDS-polyacrylamide gel.

EMSA

Synthetic nc886 for an EMSA template was prepared by *in vitro* transcription with [α -³²P]-CTP. Purified PKR was the N-terminal segment (amino acid coordinates 1-184 of NP_002750.1) containing two dsRNA binding domains (dsRBM1-2). dsRBM1-2 was overexpressed from pET-23a plasmid (Novagen, Gibbstown, NJ) in *E. coli* and was purified on a Ni-NTA column (Qiagen, Valencia, CA). Purified nc886 and dsRBM1-2 were mixed in 20 μ l EMSA buffer (200 mM Tris pH7.4, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 40 units RNase inhibitor, 2.5 μ g tRNA, 2 μ g BSA) and incubated for 15 min on ice. The samples were resolved on a 10% non-denaturing polyacrylamide gel.

Protein synthesis assay

Subconfluent cells (~10⁶) in a 6-cm dish were treated with 4 μ Ci of [³⁵S]-methionine in 2 ml of Dulbecco's Modified Eagle Medium (without L-glutamine, L-methionine, and L-cystine) containing dialyzed fetal bovine serum. After 1 hr incubation, cells were harvested and lysed. Cell lysates were precipitated with 10% trichloroacetic acid, resuspended in 1X SDS-PAGE loading buffer, and loaded onto a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue, dried, and visualized by autoradiography. Aliquots of lysates were also subjected to scintillation counting using Beckman LS 6500 Scintillation Counter to quantify incorporated [³⁵S]-methionine.

Clinical samples for qRT-PCR of nc886

The frozen liver tissues of 16 CCA patients admitted to Srinakarin Hospital (an affiliated hospital to Khon Kaen University) were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine (Khon Kaen University, Thailand). Informed consent was obtained from each subject before surgery and the research protocol (#HE521209) was approved by the Human Research Ethics Committee (Khon Kaen University). Normal bile duct epithelia were examined and taken from non-cancerous portions of CCA liver tissues.

IHC analysis for P-PKR

Eight formalin-fixed, paraffin-embedded intrahepatic CCA samples with matched normal liver tissue samples were obtained from University of Texas, MD Anderson Cancer Center. All samples were prepared in one tissue microarray block and then cut in 5 μ m-thick sections. Using the rabbit antibody against P-PKR at Thr446 (Abcam, Cambridge, MA), the immunostaining was performed by the avidin–biotin–peroxidase complex method. Antigen retrieval was performed by using citrate buffer (pH 6.0). The section was incubated with the primary antibody (1:50 dilution) for 45 minute and biotinylated anti-rabbit IgG for 30 minutes at room temperature. Finally, 3,3'-diaminobenzidine and Mayer's hematoxylin were used as a chromogen and counter staining, respectively. The P-PKR expression was interpreted by a pathologist.

Acknowledgments

We thank Dr. Sopit Wongkham, Dr. Kanlayanee Sawanyawisuth, and Ms. Sirinapa Sribenja (Khon Khan University, Thailand) for CCA cells and helpful discussion; Dr. Inhan Lee (miRcore, MI) for helpful advice; Research Histology Core Laboratory Facility, University of Texas, MD Anderson Cancer Center (funded by the National Cancer Institute Grant # CA16672) for tissue preparation and IHC. This work was supported by a Research Scholar Grant, RSG-12-187-01 – RMC from the American Cancer Society to YSL, by start-up funding from the Sealy Center for Cancer Biology at the University of Texas Medical Branch to YSL, by the Royal Golden Jubilee (RGJ) scholarship (PHD/0105/2550) of Thailand Research Fund (TRF) to NK, and the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0022022) to SHJ.

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Figure 1. Expression of nc886, vtRNA1-1, PKR, and eIF2α in cholangiocyte and CCA cells
 Top three panels: Northern hybridization of n886 and vtRNA1-1, with EtBr (ethidium bromide) staining of total RNA shown for equal loading. Bottom five panels: Western blot of PKR and eIF2α, with β-actin as a loading control. The molecular size markers in kilodalton (kD) are indicated on the right.

Figure 2. nc886 suppressed PKR in CCA cells

A. Western blot of p58IPK. The equal loading is shown in Fig 1. The expression profile of nc886, P-PKR, and P-eIF2 α from Fig 1 is summarized and indicated on the bottom. All other descriptions are the same as in Fig 1.

B. Northern hybridization of nc886 and Western blot of indicated proteins. MMNK1 cells were harvested for sample preparation, at 48 hrs after two rounds of transfection (at 0 and 24 hrs) of each indicated anti-oligo at 50 nM. All other descriptions are the same as in Fig 1.

C. PKR pulldown assays to measure PKR/nc886 association. M156 and M214 cells were harvested at 48 hrs after one round of transfection of each indicated anti-oligo at 50 nM. Cell lysates were subjected to PKR immunoprecipitation, as described in Materials and Methods. RNAs from immunoprecipitates (designated as "IP" above the gel) and from cell lysates (designated as "input") were subjected to RT-PCR measurement of nc886, vtRNA1-1 and β -actin mRNA. For comparison, genomic DNAs from each cell line (designated as "gen. DNA") were included in PCR reactions.

D. Western blot of indicated proteins in M156 and M214 cell lysates. Transfection conditions were the same as in panel C. All other descriptions are the same as in panel B.

E. Autoradiogram of *in vitro* PKR kinase assays using MMNK1 lysates, without (lane 1) or with 50 nM of each indicated anti-oligo (lane 2-3). PKR immunoprecipitates were pre-incubated with anti-oligos for 2 hrs, before adding [γ -³²P]-ATP to initiate the kinase reaction.

F. *In vitro* PKR kinase assays of M213 lysates, with indicated amounts of synthetic biotinylated nc886 or yeast tRNA which were incubated with PKR immunoprecipitates for 10 min before the kinase reaction.

G. EMSA gels with indicated amounts of PKR(dsRBM1-2). Identities of the bands are indicated on the right.

Figure 3. nc886 depletion provoked the canonical PKR/eIF2α pathway leading to apoptosis in cholangiocyte MMNK1 cells, but not in CCA cells

A. The same MMNK1 samples used in Fig 2B were subjected to eIF2α Western blot (upper panels) and global protein synthesis assays (lower panels). In lower panels, 10 μg of total protein was loaded onto a 10% SDS-polyacrylamide gel, and visualized by Coomassie blue staining (for total protein) and by autoradiogram (for [³⁵S]-methionine incorporation). Incorporated amounts of [³⁵S]-methionine were quantified by scintillation counting and indicated under each lane.

B. Cell proliferation assays (MTT assays) of MMNK1 cells after the same treatment in Fig 2B and panel A. Mock transfection without an anti-oligo (designated as “no anti-oligo”) was included as another negative control in addition to an anti-oligo against vtRNA to rule out any effect of vtRNA depletion on cell proliferation. All experiments were performed in triplicate from which an average and a standard deviation were calculated.

C. Western blot of apoptotic proteins in the indicated cell lines. Transfection conditions for each cell line are as described in earlier figures (Fig 2B for MMNK1, Fig 2C for M156 and M214) and all other descriptions are same as in Fig 2B.

D. eIF2α Western blot (upper panels) and global protein synthesis assays of M156 and M214 cells. All other descriptions are same as in panel A and C.

A **B**

Figure 4. P-PKR induced by dsRNA activated the NF- κ B branch but not the eIF2 α branch in CCA cells

A. Western blot of indicated proteins (upper panels) and global protein synthesis assays (lower panels). Cells were harvested at 24 hrs after transfecting 0.2 μ g/ml of Poly(I:C) or yeast tRNA. All other descriptions are same as in Fig 3A.

B. The NF- κ B activity was measured by luciferase assays. Assays were performed at 24 hrs after transfection of luciferase plasmids combined with 0.2 μ g/ml of Poly(I:C) or yeast tRNA. At the time of transfection, 2-AP (2-aminopurine in ethanol) was added to indicated samples at a final concentration of 2.5 mM and the corresponding volume of ethanol (vehicle) was added to the other samples. The relative luciferase values (y-axis) were calculated through several normalizations. First, firefly luciferase (*Pp*) values from pNF- κ B-Luc were normalized to Renilla luciferase (*Rr*) values from co-transfected pRL-SV40. The *Pp/Rr* values of the NF- κ B reporter plasmid were subjected to the second normalization to *Pp/Rr* values of the CMV promoter from pcDNA3.1-Zeo(+)-Pp, yielding relative *Pp/Rr* values (y-axis). The values of yeast tRNA were set as 1. For each sample, an average and a standard deviation were calculated from triplicate reactions.

Figure 5. NF- κ B was elevated by P-PKR in CCA cells and played a pro-proliferative role therein

A. *In vitro* PKR kinase assays (top panel) of indicated cell lines. Aliquots of immunoprecipitates were subjected to PKR Western blot (using antibody against total PKR) to confirm comparable pull-down efficiencies among cell lines (bottom panel).

B. NF- κ B activity measured by luciferase assays. The relative *Pp/Rr* value of MMNK1 was set as 1. All other descriptions are the same as Fig 4B.

C. qRT-PCR measurement of NF- κ B target genes (IL-8, A20, and I κ B α) and 18S rRNA as a control for equal RNA quantities. Ct (cycle threshold) values were converted to “relative cDNA amounts” by a regression line of titrating amounts of cDNA (a pool of cDNA from MMNK1, M214, and M213) and their Ct values. An average and a standard deviation were calculated from triplicate samples. For each mRNA, the value of MMNK1 was set as 1.

D. qRT-PCR measurement of NF- κ B target genes (A20, IL-6, and IL-8). M156 and M214 cells were harvested for RNA preparation, at 48 hrs after transfection of each indicated anti-oligo and/or siRNA at 50 nM. Data processing was done as described in panel C, except that values of each mRNA (“relative cDNA amounts”) were normalized to values of 18S rRNA (x-axis) and normalized values of “no transfection” was set as 1 for each mRNA.

E. Cell proliferation (MTT) assays at 24 hrs after treating CAPE, a chemical inhibitor for NF- κ B. An average and a standard deviation from triplicate samples are shown.

F. BrdU incorporation assays were done after treating 2-AP, a chemical inhibitor for PKR. All other descriptions are the same as panel E.

Figure 6. The intrinsic expression of P-eIF2α, which was independent of P-PKR, was bypassed by eIF2B overexpression in CCA cells
A. Western blot of indicated proteins after PKR knockdown. Cells were harvested at 48 hrs after transfection of indicated siRNAs at 50 nM. siGFP (targeting 5'-GGCTACGTCCAGGAGCGCA-3') was included as a negative control.
B. Western blot of eIF2B-ε, β-actin, and MetAP2(p67). All descriptions are the same as Fig 2A.

A

B

C

MDA sample #		#1	#2	#3	#4	#5	#6	#7	#8
score	CCA tumor	3+	3+	3+	3+	2+	2+	2+	2+
	matched normal	2+	2+	2+	2+	2+	2+	1+	1+

Figure 7. nc886/P-PKR expression in clinical specimens from CCA patients

A. qRT-PCR measurement of nc886 was performed in 16 pairs of cancerous and non-cancerous portion of CCA liver tissues (samples KK#1-#16 from Khon Khan University). Ct values were converted to “equivalent ng of genomic DNA” by a regression line of titrating amounts of genomic DNA and their Ct values. The converted values of nc886 were normalized to those of snU6 (y-axis). An average and a standard deviation were calculated from triplicate reactions. Samples were sorted into three groups (increased, not changed, and decreased), with a cutoff p value < 0.05.

B. IHC of P-PKR. A representative pair (MDA patient #3) is shown among eight samples from MD Anderson Cancer Center (MDA samples #1-8 in panel C).

C. The IHC signal of P-PKR was scored in a semi-quantitative fashion based on the staining intensity; +3 (strong), +2 (moderate) or +1 (weak). In case of five pairs (patient # 1, 2, 3, 5, 6), bile duct areas of matched normal tissues were used as “matched normal”. In the other three cases (patient # 4, 7, 8), matched normal bile duct tissues were unavailable and thus normal liver tissues from the same patient were used as “matched normal”.

Figure 8. Summary of data depicting the tumor surveillance model

Cell lines are in brackets. Black arrows: active pathways; grey arrows: inactivated pathways.

Cell lines used in this study

KKU-M213L0H and KKU-M213L5H are subclones from KKU-M213, with low and high Lewis-a expression respectively (personal communication with Dr. Sopit Wongkham at Khon Khan University, Thailand). Fig 1 data are summarized and tabulated.

Table 1

Cell line	Histological type	Reference	Summarized from Fig 1				
			nc886	PKR	P-PKR	eIF2α	P-eIF2α
MMNK1	immortalized cholangiocyte	(Maruyama et al. 2004) Transplantation 77(3): 446-451	+	+	-	+	-
KKU-M156	moderately differentiated adenocarcinoma	(Tepsiri et al. 2005) World J Gastroenterol 11(18): 2748-2753	+	+	-	+	+
KKU-M214	moderately differentiated adenocarcinoma	(Tepsiri et al. 2005) World J Gastroenterol 11(18): 2748-2753	+	+	+	+	+
KKU-M055	poorly differentiated adenocarcinoma	(Tepsiri et al. 2005) World J Gastroenterol 11(18): 2748-2753	+	+	+	+	+
KKU-M213	well differentiated adenocarcinoma	(Yonglithipagon et al. 2010)	-	+	+	+	+
KKU-M213L0H	metastatic clone from KKU-M213	Personal communication with Dr. Sopit Wongkham	-	+	+	+	+
KKU-M213L5H	metastatic clone from KKU-M213	Personal communication with Dr. Sopit Wongkham	-	+	+	+	+
KKU-M139	squamous cell carcinoma	(Yonglithipagon et al. 2010) Int J Parasitol 40(10): 1203-1212	-	+	+	+	+