PCAT-1 Facilitates Breast Cancer Progression via Enhancing Oxygen-independent Stability of Hypoxia-inducible Factor-1 α

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Research

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Abstract

Background: Hypoxia induces a series of cellular adaptive responses that enable to promote inflammation and cancer development. However, only few have been fully characterized about the roles of long noncoding RNAs (lncRNAs) in hypoxia-associated cancer progression.

Methods: The involvement of lncRNAs in hypoxia-related cancer progression was screened by qRT-PCR. Based on the public databases and integrating bioinformatics analysis, the alteration of prostate cancer associated transcript-1 (PCAT-1) in breast cancer tissues was detected and validated in a cohort of breast cancer tissues. Overexpression and knocking down experiments were performed to uncover the biological roles of PCAT-1 on cell hypoxia-associated phenotypes and biological behaviors. RNA immunoprecipitation (RIP) and RNA pull-down were carried out to reveal the physical interaction between PCAT-1 and receptor of activated protein-C kinase-1 (RACK1). Moreover, xenograft mouse models were used to evaluate the influence of PCAT-1 on cancer progression and metastasis in vivo.

Results: We identified PCAT-1 as a hypoxia-inducible lncRNA that regulated the hypoxia-inducible factor-1 α (HIF-1 α) stability, crucial for cancer progression. Extensive analyses of clinical data indicated that PCAT-1 was elevated in breast cancer patients and was associated with pathological grade, tumor size and poor clinical outcomes. Through gain and loss of function experiments, we found that PCAT-1 promoted hypoxia-associated breast cancer progression including growth, migration, invasion, colony formation, and metabolic regulation. Mechanistically, PCAT-1 directly interacted with RACK1 protein and prevented RACK1 from binding to HIF-1 α , thus protecting HIF-1 α from RACK1-induced oxygen-independent degradation.

Conclusions: These findings provide a new insight into lncRNA-mediated mechanisms for HIF-1 α stability and suggest that a novel role of PCAT-1 as a potential therapeutic target for breast cancer.

Background

Breast cancer is a major public health problem and it is the most commonly diagnosed cancer among women. The implementation of breast cancer early diagnosis, together with local surgery and radiotherapy, and systemic pharmacological treatments, has resulted in substantial improvement in the therapeutic effects of breast cancer [1, 2]. However, the cancer progression and the spread of cancer cells to other organs are the cause of death in the vast majority of patients, and the processes are complicated and involved many complex epigenetic changes [3, 4]. Since the pathogenic mechanisms of cancer progression are not fully understood, more studies are needed to discover and develop effective molecules and targets for the diagnosis and treatment.

Hypoxia in tumor microenvironment is associated with inflammation, tumorigenesis and therapeutic resistance [5–7]. Although hypoxia occurs at different stages of breast cancer development, it is unclear how hypoxia affects the cells during transformation. Hypoxia-inducible factor-1 (HIF-1) is a key regulator of the hypoxia response and cancer promotion [8, 9]. It is essential for the cellular response to hypoxia,

including the angiogenesis, energy metabolism, cell survival, invasion and metastasis [10, 11]. HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β subunits. HIF-1 β is maintained constitutively, whereas HIF-1 α accumulates rapidly in hypoxia and degraded under normoxic conditions. In normoxia, HIF-1 α is degraded by oxygen-dependent prolyl hydroxylation, ubiquitination, and proteasomal degradation [11]. Under hypoxic condition, HIF-1 α evades the oxygen-dependent degradation, allowing it to translocate into the cell nucleus to form the transcriptionally HIF-1 α complex [10]. Several studies have shown that there are oxygen-independent mechanisms about HIF-1 α degradation under hypoxic conditions, including heat shock protein 90 (HSP90) and receptor of activated protein C kinase-1 (RACK1) [12–14]. HSP90 binds to the HIF-1 α PAS domain to prevent its degradation. RACK1 competes with HSP90 for binding to HIF-1 α , links HIF-1 α to Elongin-C, and promotes HIF-1 α degradation in an oxygen-independent manner [12, 15]. Thus, equilibrium between HIF1 α /RACK1 and HIF1 α /HSP90 may play a key role in maintaining HIF1 α stability, as well as cancer progression.

Long non-coding RNAs (lncRNAs) have emerged as important molecules in carcinogenesis with various biological effects, such as genome modification, transcriptional activation, and transcriptional interference [16–18]. They have been implicated as prospective biomarkers for early cancer diagnosis, with the potential to serve as new targets for cancer treatment [19, 20]. Previous studies have identified lots of hypoxic-reactive lncRNAs are functionally characterized and have crucial effects on cancer progression [21]. Prostate cancer associated transcript-1 (PCAT-1) is identified as a prostate cancer-overexpressed lncRNA, and it contributes to prostate cancer progression through regulation of target genes [22]. The high expression of PCAT-1 has been observed in several types of cancers, including colorectal cancer [23], esophageal squamous cell carcinoma [24] hilar cholangiocarcinoma [25], osteosarcoma [26] and hepatocellular carcinoma [27]. It has been shown to be involved in impairment of double-stranded DNA break repair, cancer cell proliferation, epithelial-mesenchymal transitions, migration, and invasion [28–31]. The recent report shows that PCAT-1 overexpression is also observed in breast cancer tissues [32]. However, there is no previous report of PCAT-1 regulating hypoxia-related cancers. Currently, the clinical significance of PCAT-1 in the context of breast cancer and the molecular mechanisms by which it regulates remain largely unclear.

In this study, through the investigation of lncRNA in breast cancer, we identify that PCAT-1 is one of the significantly upregulated lncRNA under hypoxic conditions. We reveal that PCAT-1 is critical for maintaining the stability of HIF-1 α in hypoxic breast cancer cells, which is related to advanced disease progression and poor prognosis. We elucidate that PCAT-1 interacts with RACK1, which mediates oxygen-independent HIF-1 α stability, thereby establishing PCAT-1 as an important lncRNA in the breast cancer.

Methods

Cell culture and transfection

Human breast cancer cell lines MCF-7, MDA-MB-231, normal breast epithelial cell MCF10A and the human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection

(Manassas, VA, USA). The cell lines were cultured in DMEM (Gibco, USA) with 10% foetal bovine serum, 100 ng/ml streptomycin and 100 U/ml penicillin. For normoxic conditions, mixed gas consisting of 20% O₂, 5% CO₂, and 75% N₂ at 37°C was used. For hypoxic conditions, the cells were cultured in the gas mixture of 1% O₂, 5% CO₂, and 94% N₂ at 37°C.

Transfection of the cells with siRNAs was performed using **Lipofectamine** RNAiMAX (Invitrogen, USA) according to the manufacturers' instructions. For siRNA-mediated knockdown of PCAT-1, two different siRNA were synthesized by Genepharma (Shanghai, China) using the following sequences: si-PCAT-1 #1: GCAGAAACACCAAUGGAUAAU; si-PCAT-1 #2: GAACCUAACUGGACUUUAAU. The specific siRNAs for RACK1 was purchased from Santa Cruz Biotechnology (**Santa Cruz**, USA). The lentiviral PCAT-1 shRNA (shRNA-PCAT-1) were constructed by Hanbio (Shanghai, China) using the following sequences: AUACAUAAGACCAUGGAAAU [28]. The PCAT-1, truncated PCAT-1 (Δ 1-400), truncated PCAT-1 (Δ 401-800), truncated PCAT-1 (Δ 801-1,299) and truncated PCAT-1 (Δ 1-800) overexpressed lentiviruses were constructed by Hanbio (Shanghai, China). To express and purify GST-RACK1, a full length of RACK1 and its truncated RACK1 cDNAs were amplified by PCR using the appropriate primers, and subcloned in frame into pGEX4T-1 to make the GST fusion protein using bacterial expression vectors. The HIF1 α mutant coding plasmid HA-HIF-1 α -P402A/P564A-pcDNA3 was purchased from Addgene (Cambridge, MA, USA).

Tissue specimens

Tumors and the adjacent breast tissues used in this study were surgical specimens from patients with breast cancer. All samples were fresh frozen in liquid nitrogen and stored at -80 °C until RNA extraction. The study protocol was approved by the Ethics Committee of the Second Hospital of Hebei Medical University (Hebei, China), and informed consent was obtained from all participants.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by TRIzol reagent (Invitrogen, USA). Equal amounts of RNA were reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (**Roche**), and qRT-PCR was performed with SYBR PrimeScript RT-PCR kit (Takara, Dalian, China). Unless otherwise specified, the amplified transcript level of each specific gene was normalized against the *GAPDH* mRNA level. The primers were provided by Shenggong Company (Shanghai, China). The primer sequences are listed in Additional file 1: Table S1.

Bioinformatics Analysis

The Cancer Genome Atlas (TCGA) Breast Cancer datasets were retrieved from the cBioPortal for Cancer Genomics. PCAT1 gene log₂ copy number from cBioPortal was downloaded. Patients were grouped into PCAT1-low or PCAT1-amplification based on PCAT1 log₂ copy number. The cutoff point of PCAT1 gene

$\log_2$ copy number calls on the cases were determined as the value yielding maximum sum of sensitivity and specificity. The clinical data (including the overall survival data and disease-free survival data) were obtained from the TCGA Pan-Cancer Clinical Data Resource [33]. The patients with overall event time less than 130 months were used in this study. The survival curves were constructed according to the Kaplan-Meier method and compared using the log-rank test.

Cell growth assay

The cell growth was monitored by MTT assay. Briefly, cells were treated and cultured into 96-well plates. A total of 20 μ l of MTT (Sigma, USA) dissolved in PBS at 5 mg/ml was added to the wells and then incubated for an additional 4 h at 37°C. A total of 100 μ l of DMSO was added to dissolve the formed formazan crystals, and the optical density was measured on the microplate reader.

Colony formation assay

Cells were seeded in 6-well plates (200 cells/well) and treated for 14 days, and then the cells were fixed with methanol and stained with 1% crystal violet solution. The numbers of cell colonies from three dishes were counted.

Wound healing and invasion assay

For the wound healing assays, cells were treated and cultured in six-well plates until almost totally confluent. Then, the artificial wounds with a sterile pipette tip were scratched and images were captured at 0 h and 48 h under the microscope.

Cell invasion assays were performed using the Transwell chambers (8 μ m pores, Corning, USA) that coated with Matrigel (BD Biosciences, USA). Cells were treated and suspended in medium with 1% FBS in the upper chambers. Medium containing 10% FBS was used as a chemoattractant in the bottom chamber. After incubation for 48 h, the migrated or invaded cells were fixed, stained and counted under an inverted microscope.

Measurement of extracellular acidification rate (ECAR)

The ECAR was measured using the Seahorse Extracellular Flux Analyzer XF96 (Seahorse Bioscience, USA) according to the manufacturer's instructions. Briefly, the treated cells were seeded at a density of 10^5 per well allowed to adhere overnight. Before the assay, the cells were washed with assay medium followed by a sequential injection of 10 mM glucose, 1 μ M oligomycin, and 100 mM 2-deoxyglucose (2-DG). ECAR is expressed as mpH/min and three replicates were performed for each group.

Lactate production assay

Cells were seeded into 6-well plates, and the culture medium was collected for measurement of lactate concentration. The Level of lactate in the culture media was determined using the L-lactate Assay Kit (Abcam, USA) following the manufacturer's instruction.

Immunoblot analysis and Co-immunoprecipitation (Co-IP)

For immunoblot analysis, RIPA buffer was added and incubated for 30 min followed by centrifugation, the supernatant contained the total protein. The nuclear and cytoplasmic proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA) according to the manufacturer's instructions. After determination of the protein concentrations by the Bradford method, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% fat-free milk powder in TBST buffer and then exposed to primary antibodies at 4 °C overnight. Following incubation with secondary antibodies, signals were detected using the ECL detection system.

For immunoprecipitation, protein extracts from the cells were determined by the Bradford method. For the experiment, 500 µg of protein immunoprecipitated with indicated antibodies at 4°C overnight, followed by incubation with protein A Sepharose beads for 2 h. The Immunoprecipitates were washed and resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

For co-IP, cell lysates were mixed with mouse/rabbit IgG or primary antibodies, and then the antibody-protein complex was following incubated with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, USA). The agarose-antibody-protein complex was collected and then assessed by immunoblot analysis. The following antibodies were used: anti-HIF-1 α (Abcam), anti-RACK1 (Cell Signaling), anti-HSP90 (Abcam), anti-p-Ser (Abcam), anti-HA (Abcam), anti-FLAG (Sigma), anti- β -actin (Abcam).

RNA pull-down assay

Biotin-labeled PCAT-1 and its fragments were transcribed with Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Invitrogen, USA), treated with RNase-free DNase I (Invitrogen, USA), and purified using RNeasy Mini Kit (Qiagen, Germany). Pull downs were performed with biotinylated RNA and nuclear extracts of cells. The bead-RNA-protein complexes were isolated with streptavidin magnetic beads (Sigma, USA) and subjected to standard immunoblot analysis.

RNA immunoprecipitation (RIP)

The RIP assay was performed using the Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore, USA). Briefly, cells were harvested and lysed in lysis buffer containing protease inhibitor

cocktail and RNase inhibitors. Cell extracts were co-immunoprecipitated using the primary antibody, and the retrieved RNA was subjected to qRT-PCR analysis to demonstrate the presence of the binding products using respective primers.

Xenograft experiments

The animal studies were performed as described previously [34, 35]. The female BALB/c nude mice (4-5 weeks old) were purchased from Charles River (Beijing, China). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Second Hospital of Hebei medical University (Hebei, China). A total of 10^6 MDA-MB-231 cells (transfected with sh-control or sh-PCAT-1) were injected into the right flank or the tail vein of mice ($n = 6$ mice/group), respectively. The tumor volume was measured every 5 days, and tumor volume was estimated by the formula: $V(\text{mm}^3) = 1/2 \times \text{length} \times \text{width}^2$. At the end of the experiment, the tumor tissues in the tumor growth xenograft model and the lungs and livers in the metastasis model were removed and weighed.

Statistical analysis

The overall or disease-free survival curves were calculated by the Kaplan-Meier method, and the differences were assessed using the log-rank test. Differences between two independent groups were tested with two-tailed Student's t test. Comparison of multiple groups was made using a one- or two-way ANOVA tests. Univariate and multivariate Cox proportional hazards regression models were performed to explore independent prognostic factors. Data were expressed as mean $\pm$ s.e.m. Difference was considered statistically significant at $P < 0.05$.

Results

The expression of PCAT-1 is induced after hypoxia treatment

To identify the involvement of lncRNAs in hypoxia-related cancer progression, we selected 30 cancer-associated lncRNAs (listed in Additional file 1: Table S2) according to the previous studies [20, 21] and analyzed their expression in MDA-MB-231 breast cancer cells in hypoxia and normoxia. Compared to normoxic conditions, 10 lncRNAs with >2-fold expression changes were identified under hypoxic conditions (Fig. 1a). Moreover, we examined the expression of these lncRNAs and identified 7 of them upregulated by >2-fold in MDA-MB-231 cells treated with cobalt chloride (CoCl_2) (Fig. 1b), which was used to create a *pseudo-hypoxic* environment. These lncRNAs include 4 of those induced in physical hypoxia (Fig. 1c). PCAT-1 was one of the upregulated lncRNA after cobalt chloride treatment and showed a time- and concentration-dependent induction (Fig. 1d and e). Similarly, a time-dependent increase of PCAT-1

was observed in MDA-MB-231 cells under hypoxic conditions (Fig. 1f), confirming PCAT-1 is a hypoxia-responsive lncRNA and is probably involved in HIF-1 α signalling pathway.

PCAT-1 expression is associated with breast cancer poor prognosis

To assess the pathological and clinical value of PCAT-1 in breast cancer, we measured PCAT-1 expression in a panel of 71 matched pairs of clinical specimens containing breast cancer tissues and the normal adjacent tissues. It showed that PCAT-1 was significantly upregulated in the cancer tissues compared with the normal adjacent tissues ($P < 0.001$, Fig. 1g). Moreover, statistical analysis showed that PCAT-1 overexpression was strongly correlated with the advanced tumor stage ($P = 0.001$, Fig. 1h) and tumor size ($P = 0.003$, Fig. 1i).

To further validate the data from our studies, we surveyed public data of The Cancer Genome Atlas (TCGA) breast cancer study using cBioPortal platform and explored the correlation between PCAT-1 gene alteration and the clinicopathological characteristics of 735 breast cancer patients (Additional file 1: Table S3). The analyses revealed that PCAT1-amplification was correlated with advanced tumor stage ($P = 0.008$) and tumor sizes ($P = 0.002$, Table 1), whereas no significant relationship with any other clinical characteristics was observed. Moreover, we evaluated PCAT-1 gene alteration in relation to overall survival (OS) and disease-free survival (DFS) data on the patients (Additional file 1: Table S3). Kaplan-Meier survival analysis was performed to compare the outcomes of patients dichotomized by PCAT log₂ copy number value. Patients with PCAT1-amplification copy number had a significantly worse overall survival ($P < 0.001$) (Fig. 1j) and disease-free survival ($P = 0.004$) (Fig. 1k), respectively. In addition, similar to the prognostic values of tumor stage (I+II versus III+IV), tumor size (≤ 5 cm versus > 5 cm) and lymph node metastasis (N0 versus N1-3), the univariate Cox proportional hazards showed that PCAT1-amplification was another strong prognostic predictor for poor OS (hazard ratio=0.423, $P < 0.001$) and DFS (hazard ratio=0.488, $P = 0.005$, Table 2). Furthermore, multivariate analysis was conducted to determine risk assessment related to OS and DFS. The PCAT1-amplification was identified as an independent prognostic factor for OS (HR=0.497, $P = 0.005$, multivariate analysis) and DFS (HR=0.556, $P = 0.024$, multivariate analysis, Table 2). Together, our data indicated that PCAT1 could be used as an independent prognostic indicator of clinical prognosis in breast cancer patients.

Table 1

The relationship between PCAT-1 gene alteration and clinicopathological characteristics in breast cancer patients.

Clinicopathologic characteristics	PCAT-1 gene alteration		Number of cases	P-value
	Amplification	Low		
Age(years)				
≤60	122	305	427	0.742
>60	92	216	308	
Tumor stage				
I+II	61	100	161	0.008**
III-IV	153	421	574	
Tumor size				
≤5cm	41	157	198	0.002**
>5cm	173	364	537	
Lymph node metastasis				
N0	97	277	374	0.062
N1-3	117	244	361	
Fisher's exact test was used to determine the correlation between PCAT-1 gene alteration and the clinicopathological characteristics. The cutoff point is set as the value yielding maximum sum of sensitivity and specificity for disease-free survival analysis. **P<0.01.				

Table 2

Univariate and multivariate Cox regression analyses of overall survival and disease-free survival in breast cancer patients.

Covariate	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Overall survival						
Age (≤ 60 versus >60)	1.826	1.122-2.971	0.015*	2.170	1.316-3.579	0.002**
Tumor stage (I+II versus III+IV)	0.360	0.220-0.589	0.000**	0.463	0.248-0.864	0.016*
Tumor size (≤ 5 cm versus >5 cm)	2.327	1.217-4.448	0.011*	1.701	0.868-3.332	0.122
Lymph node metastasis (N0 versus N1-3)	2.132	1.284-3.537	0.003**	1.345	0.710-2.548	0.363
PCAT-1 gene alteration (low versus amplification)	0.423	0.261-0.686	0.000**	0.497	0.305-0.811	0.005**
Disease-free survival						
Age (≤ 60 versus >60)	0.817	0.478-1.396	0.459	0.906	0.526-1.560	0.721
Tumor stage (I+II versus III+IV)	0.322	0.194-0.535	0.000**	0.331	0.166-0.658	0.002**
Tumor size (≤ 5 cm versus >5 cm)	2.089	1.086-4.017	0.027*	1.491	0.751-2.957	0.253
Lymph node metastasis (N0 versus N1-3)	1.707	1.021-2.854	0.042*	0.817	0.407-1.637	0.568
PCAT-1 gene alteration (low versus amplification)	0.488	0.295-0.808	0.005**	0.556	0.334-0.926	0.024*
HR, hazard ratio; 95% CI, 95% confidence interval. *P<0.05 and **P<0.01.						

PCAT-1 promotes breast cancer progression

To ascertain the potential role of PCAT-1 in breast cancer, we detected the expression of PCAT-1 in a panel of breast cancer cells MCF-7, MDA-MB-231 and normal breast epithelial cell MCF10A. Compared with MCF-7 cells that exhibited poor invasive capacity, the high invasive MDA-MB-231 cells had higher PCAT-1 expression (Fig. 2a), while MCF-7 and MCF10A cells had low level of PCAT-1, indicating PCAT-1 was

possible association with cell invasion in breast cancer. Similar to MDA-MB-231 cells, PCAT-1 expression was also induced in MCF-7 cells by cobalt chloride *under* pseudohypoxia or under hypoxic conditions (Fig. 2b).

The cells response to pathological hypoxia is associated with many critical aspects of cancer. To further investigate the biological significance of PCAT-1 in breast cancer, we knocked down the expression of PCAT-1 with two independent small interfering RNAs (siRNAs). Moreover, lentiviral PCAT-1 shRNA (shRNA-PCAT-1) and empty vector (shRNA-Control) were also stably transfected in MDA-MB-231 cells, and the related phenotypes in the cells were examined. The functional assays revealed that PCAT-1 knockdown significantly impaired cell growth, migration, invasion and colony formation in hypoxia (Fig. 3c-f). In addition, we found the hypoxia-induced extracellular acidification rate (ECAR) (Fig. 2g) and lactate production (Fig. 2h) were significantly reduced in the PCAT-1-knockdown MDA-MB-231 cells under hypoxic conditions, indicating that PCAT-1 is essential for glycolytic metabolism in the cells. In conclusion, these results suggested that PCAT-1 played a crucial role in hypoxia-associated phenotypes and biological behaviors.

PCAT-1 contributes to HIF-1 α stability

HIF-1 α is a core regulator of cell adaptation to hypoxia. To determine the roles of PCAT-1 in HIF-1 α signalling pathway, PCAT-1 was overexpressed in MCF-7 cells. We found that HIF-1 α protein expression was increased in normoxia as well as in hypoxia (Fig. 3a). However, *HIF-1 α* mRNA expression was not changed (Fig. 3b). In addition, we investigated whether PCAT-1 regulates *HIF-1 α* mRNA stability. We treated the cells with actinomycin D, which blocks *de novo* mRNA synthesis, and the *HIF-1 α* mRNA stabilization is not affected by PCAT-1 overexpression (Fig. 3c), suggesting that *HIF-1 α* mRNA transcription and degradation is not regulated by PCAT-1. Additionally, the cells were exposed to hypoxia, and then recovered in normoxia, and we found that the half-lives of HIF-1 α protein were increased from 2.5 ± 0.3 to 6.5 ± 0.7 min as a consequence of PCAT-1 overexpression, indicating that PCAT-1 overexpression yielded a significantly delay of HIF-1 α protein degradation (Fig. 3d). These results reveal that PCAT-1 overexpression increases HIF-1 α expression by enhancing the stability of HIF-1 α protein. In addition, PCAT-1 was depleted in MDA-MB-231 cells. HIF-1 α protein expression was obviously decreased in the cells after knockdown of PCAT-1 under hypoxic environment (Fig. 3e), while *HIF-1 α* mRNA expression and stabilization were not changed (Fig. 3f). We also found that HIF-1 α protein degraded more quickly during reoxygenation in the PCAT-1 knockdown cells (Fig. 3g), and the half-lives of HIF-1 α protein were decreased from 7.2 ± 1.1 to 3.8 ± 0.6 min, suggesting that depletion of PCAT-1 decreased the HIF-1 α protein stability. Moreover, PCAT-1 knockdown reduced HIF-1 α nuclear accumulation, which is consistent with the reduction of HIF-1 α protein stability (Fig. 3h).

Furthermore, to determine whether PCAT-1 modulated HIF-1 α activity, we investigated the expression of HIF-1 α target genes such as *Flt1*, *Vegfa*, *ANGPTL4*, *Ccng2*, *Sox9*, *Pdk1*, *Plod2*, *Ldha*, GLUT1 and BNIP3 in the cells. PCAT-1 knockdown attenuated the expression of these genes in MDA-MB-231 cells in hypoxia,

without a significant change of the gene expression in normoxia (Fig. 3i). Reciprocally, PCAT-1 overexpression in MCF-7 cells induced the expression of HIF-1 α target genes in hypoxia (Fig. 3j). Collectively, these results indicate a crucial role of PCAT-1 in HIF-1 α protein stability and activity under hypoxic conditions.

PCAT-1 protects HIF-1 α from RACK1-mediated oxygen-independent degradation

To determine whether the effects of PCAT-1 on HIF-1 α protein stability related to prolyl hydroxylase, HA-HIF-1 α (P402A/P564A), which contained the proline-to-alanine substitutions and conferred irresponsiveness to [hydroxylation](#) [15], was transfected in MDA-MB-231 cells. We found that PCAT-1 knockdown enhanced mutant HIF-1 α degradation in hypoxia, revealing an independence of prolyl hydroxylation (Fig. 4a). Additionally, PCAT-1 overexpression increased HIF-1 α protein stability in MCF-7 cells cotransfected with HA-HIF-1 α (P402A/P564A) and PCAT-1 under hypoxic conditions (Fig. 4b). Then MDA-MB-231 cells were cotransfected with PCAT-1 siRNA and RACK1 siRNA, and we found that RACK1 knockdown alone accumulated HIF-1 α expression in the cells. However, HIF-1 α expression reduced when *PCAT-1* was silenced at the same time (Fig. 4c). Moreover, we found that RACK1 knockdown notably improved HIF-1 α protein stability in MCF-7 cells cotransfected with PCAT-1 and HA-HIF-1 α (P402A/P564A) (Fig. 4d). In addition, PCAT-1 was overexpressed in MCF-7 cells and then the cells were treated with HSP90 inhibitors 17-AAG, and the results showed that PCAT-1-facilitated HIF-1 α protein stability was abolished after 17-AAG treatment (Fig. 4e). Moreover, PCAT-1 knockdown in MDA-MB-231 cells accelerated HIF-1 α degradation after 17-AAG treatment (Fig. 4f). Taken together, these results demonstrated that PCAT-1 played an important role in the function of RACK1 and HSP90 on HIF-1 α protein stability.

PCAT-1 interacts with RACK1 that mediates HIF-1 α stability

RACK1 could compete with HSP90 for binding to HIF-1 α and be involved in HIF-1 α degradation [12]. Given the established interaction of RACK1 with HIF-1 α , we sought to analyze the mechanistic role of PCAT-1 in HIF-1 α stability by focusing on the interaction between PCAT-1 and RACK1. We first explored the possible interaction between PCAT-1 and RACK1 through bioinformatic analysis (catRAPID), which could predict the protein-RNA associations through estimating the interaction propensity between amino acids and nucleotides by secondary structure information, hydrogen bonding and Van der Waals forces. Results from catRAPID fragments analysis showed that the 1093-1172 and 1288-1367 nucleotide positions of the PCAT-1 sequence may bind to the 217-268 amino acid residues of the RACK1 protein with high affinity (Fig. 5a).

To validate the physical interaction between PCAT-1 and RACK1, we performed RNA pull-down followed by immunoblot using RACK1 antibody in MDA-MB-231 cells. The results indicated that PCAT-1 was able

to interact with RACK1 (Fig. 5b). Consistently, RIP assays using nuclear extract further confirmed that PCAT-1 directly interacted with RACK1 (Fig. 5c). Moreover, a series of *PCAT-1* truncated fragments analysis demonstrated that the 3'-terminal (nucleotides 801-1299) region of the PCAT-1 were required for direct interaction with RACK1, which was confirmed by RNA pull-down assays (Fig. 5d). As expected, the interaction was abrogated when using the truncated *PCAT-1* (nucleotides 1-800) that lacked the binding region (Fig. 5e), proving that the critical region of *PCAT-1* at the 3'-terminal (nucleotides 801-1299).

To further clarify the functional relevance of the *PCAT-1*/RACK1 interaction, the full-length and truncated *PCAT-1* (nucleotides 1-800) were overexpressed in MCF-7 cells. The results showed that, compared to the full-length PCAT-1, the ability of RACK1 binding-deficient mutant PCAT-1 (nucleotides 1-800) to maintain HIF-1 α stability and induce HIF-1 α target genes was significantly impaired (Fig. 5f and g). Moreover, overexpression of the truncated *PCAT-1* (nucleotides 801-1299) in PCAT-1-silenced MDA-MB-231 cells restored the hypoxia-associated cancer phenotypes in the cells (Fig. 5h-k). Full-length RACK1 consists of seven tandemly repeated WD40 domains. To map which WD40 domain on Rack1 is required for *PCAT-1* binding, we generated several deletion mutants of RACK1 consisting of WD2-7, WD3-7, WD4-7, WD5-7, WD6-7 and WD7. The RIP assays revealed that constructs containing the sixth WD40 domain could bind to PCAT-1 (Fig. 5l), suggesting that the sixth WD40 domain was responsible for the *PCAT-1*/RACK1 interaction. These data suggest that the PCAT-1 and RACK1 binding region functionally contribute to the *PCAT-1*-mediated HIF-1 α protein stability.

PCAT-1 reduces RACK1 binding to HIF-1 α

To evaluate the effect of *PCAT-1* on RACK1 binding to HIF-1 α , MDA-MB-231 cells transfected with shRNA-PCAT-1 were treated with MG132. Immunoprecipitation results showed that PCAT-1 knockdown reduced HIF-1 α binding to HSP90 but significantly increased the binding efficiency between HIF-1 α and RACK1 (Fig. 6a). Moreover, MCF-7 cells were cotransfected with PCAT-1 and HA-HIF-1 α (P402A/P564A) under hypoxic conditions. The results showed that PCAT-1 overexpression significantly suppressed HA-HIF-1 α binding to RACK1 (Fig. 6b). Moreover, HSP90 inhibition by 17-AAG increased the binding efficiency between RACK1 and HIF-1 α , while it can be abolished by PCAT-1 overexpression. In addition, we investigated the effect of PCAT-1 on RACK1 dimerization which is necessary in HIF-1 α **activation** [36]. FLAG-RACK1 was overexpressed in HEK 293 cells, and then it was isolated by anti-FLAG antibody immobilized on agarose resin. We further used the FLAG-RACK1-enriched resin to pull down endogenous RACK1 from the MDA-MB-231 cells transfected with shRNA-PCAT-1, and the results showed that PCAT-1 knockdown increased RACK1 dimerization in the presence of MG132 under hypoxic conditions (Fig. 6c). Consistent with the previous study that serine phosphorylation of RACK1 **was required for its dimerization and HIF-1 α degradation** [36], we found that PCAT-1 knockdown also enhanced RACK1 phosphorylation (Fig. 6d). These results suggest that PCAT-1/RACK1 interaction results in decreased RACK1 dimerization and phosphorylation, which in turn reduces RACK1 binding to HIF-1 α .

Suppression of breast cancer progression by targeting PCAT-1 in vivo

To evaluate the biological function of PCAT-1 in vivo, MDA-MB-231 cells stably transfected with shRNA-PCAT-1 or shRNA-Control lentiviral particles were inoculated into the right flank of nude mice (n=8/group). Five weeks later, compared to shRNA-Control, the shRNA-PCAT-1 tumor growth was dramatically delayed (Fig. 7a-c).

We further injected the cells into the tail veins of nude mice in order to evaluate the metastatic potential of the cells. The mice were sacrificed 8 weeks later and the lung and liver tissues were obtained. We found that the metastasis ability of MDA-MB-231 cells to lung and liver in the PCAT-1 silencing group were significantly lower relative to the control group (Fig. 7d and e). Taken together, these results indicated that PCAT-1 knockdown significantly suppressed the breast cancer progression and metastasis in vivo, which further confirmed the carcinogenic effects of PCAT-1 in breast cancer.

Discussion

Hypoxia is common in solid tumors, and it modulates cancer progression as well as therapeutic response. Recently, more and more observations recognize that lncRNA can act as a regulator of cellular responses in hypoxia. Under hypoxic conditions, lncRNA plays the regulatory roles through a variety of potential mechanisms such as epigenetics, lncRNA-miRNA interaction, and lncRNA-protein interaction [37–39]. However, only a few have been confirmed in terms of the detailed mechanisms, while the roles of most others are still unclear.

Previous studies have implicated the elevated PCAT-1 expression is observed in breast cancer [32]. However, none of the studies have investigated the biological functions of PCAT-1 in the hypoxic environment which is contributed to the malignant progression. Our present study identifies PCAT-1 as a hypoxia-inducible lncRNA and shows a distinct mechanism in breast cancer. Indeed, we find that PCAT-1 overexpression enhances HIF-1 α protein stability and confers hypoxia-associated tumorigenesis including growth, migration, invasion, colony formation, and metabolic regulation. When we investigate the mechanisms of PCAT-1 promoting breast cancer under hypoxic conditions, we find that RACK1 is involved and plays a key role. As a multifunctional scaffold protein for many kinases and receptors, RACK1 binds to the PAS-A subdomain of HIF-1 α and recruits an Elongin-C E3 ubiquitin ligase complex to HIF-1 α , enhancing HIF-1 α ubiquitination and degradation. Here, we show that PCAT-1 is capable of controlling the competition of RACK1 for binding to HIF-1 α . The interaction between PCAT-1 and RACK1 inhibits RACK1 dimerization and phosphorylation, and then further prevents RACK1 from binding to HIF-1 α . The binding of RACK1 to HIF-1 α was disrupted by PCAT-1, making HIF-1 α easier to bind to HSP90, but not to RACK1, and contributing to enhance HIF-1 α stability.

As the expression of PCAT-1, RACK1, HSP90 and HIF-1 α is increased in many kinds of cancers [30, 40–42], the effects of PCAT-1 on the balance between HIF-1 α /RACK1 and HIF-1 α /HSP90 may contribute to

the cancer progression. It is the basis for the association of PCAT-1 with the clinicopathological prognosis of the diseases. As we have shown, PCAT-1 is almost absent or at low level in the normal tissues, and it increased to high level in the tumor tissues. Considering the role of PCAT-1 in the upregulation of HIF-1 α , facilitation of HIF-1 α stability by PCAT-1 may explain the close relation of PCAT1 overexpression to advanced tumor stage and tumor size. In addition to hypoxia, PCAT-1 also promotes the expression of HIF-1 α in normoxia. Since HIF-1 α also increases in some cancer cells under normoxic conditions [43], it can be speculated that PCAT-1 also participates in the regulation of HIF-1 α signaling pathway in normoxia during cancer progression.

It is demonstrated that HIF-1 α and its target genes control the biological processes in cancers [42, 44, 45]. HIF-1 α and the downstream effectors have been considered as potential targets for cancer diagnosis and therapy due to their profound impacts in cancers [11, 46]. However, it is hard to design the effective inhibitors due to the complexity of the HIF-1 α signaling network [45]. Because lncRNA is easy to extract and detect, it has many advantages in cancer diagnosis and prognosis [19]. Our works present the evidence showing that PCAT-1 is an independent risk factor for OS and DFS, suggesting the potential clinical application of PCAT-1 as a biomarker for breast cancer prognosis. Thus, these findings illustrate that PCAT-1 intervention through gene silencing or other strategies could present potential new approach to anticancer therapies.

Although our study supports that the expression of PCAT-1 is critical to the breast cancer, PCAT-1 overexpression may be the result of other modulations in cells, and it may be regulated by other factors involved in cancer progression. Our research can not completely rule out the possibility that PCAT-1 may also enhance the HIF-1 α stability through regulating other factors that affect HIF-1 α degradation. For example, some post-translational modifications, such as acetylation, phosphorylation, nitrosylation and SUMOylation, have also been reported to be involved in the regulation of transactivation, stability and expression of HIF-1 α [47]. These questions should be solved through future experiments to clarify the role and mechanism of PCAT-1 in breast cancer.

Conclusions

In summary, our studies highlight a previously unknown function of PCAT-1 in the cancer progression under hypoxic conditions, especially its role in regulating the oxygen-independent stability of HIF-1 α . The effects of PCAT-1 in HIF-1 α stability not only provide new insight into the survival mechanism of cells in response to oxygen deficiency but also reveal a potential utility of PCAT-1 in prognosis and therapeutic strategy for breast cancer.

Abbreviations

RACK1: Activated protein-C kinase-1; Co-IP: Co-immunoprecipitation; DFS: Disease-free survival; ECAR: Extracellular acidification rate; HSP90: Heat shock protein 90; HIF-1 α : Hypoxia-inducible factor-1 α ; PCAT-

1: Prostate cancer associated transcript-1; qRT-PCR: Quantitative real-time PCR; RIP: RNA immunoprecipitation; TCGA: The Cancer Genome Atlas; OS: Overall survival

Declarations

Ethics approval and consent to participate

All human breast cancer tissues and the adjacent breast tissues used in this study were approved by the Ethics Committee of the Second Hospital of Hebei Medical University, and informed consent was obtained from all participants. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Second Hospital of Hebei medical University.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of data and materials

All data generated or analysed during this study are available on published databases TCGA.

Competing interests

The authors declare no competing interests.

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Authors' contributions

BL and JG designed the study. JW, XC, NZ and MY performed the experiments. JW and XC collected clinical samples. NZ, MY and JG analyzed the data. JG and BL wrote the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

PCAT-1 is involved in the response to hypoxia and related to poor prognosis for breast cancer. Expression of 30 lncRNAs in MDA-MB-231 cells under hypoxia (1% O₂, 6 h) (a) or treated with cobalt chloride (200 μM, 6h) (b) was quantified by qRT-PCR and normalized to GAPDH expression. c The Venn diagram illustrated the number of lncRNAs elevated in hypoxia or treated with cobalt chloride. Expression of PCAT-1 in MDA-MB-231 cells treated with cobalt chloride (200 μM) for different times (d) or with different concentrations of cobalt chloride for 24 h (e). f Expression of PCAT-1 in MDA-MB-231 cells under hypoxic conditions. g PCAT-1 expression in 71 pairs of breast cancer tissues and the normal adjacent tissues was detected by qRT-PCR and normalized to GAPDH expression. PCAT-1 expression in breast cancer tissues (n = 71) assessed by qRT-PCR with tumor stage (h) and tumor size (i). Kaplan-Meier curve for OS (j) and DFS (k) in breast cancer patients with and without genetic amplification of PCAT-1. Patients were grouped

into PCAT1-low or PCAT1-amplification based on the PCAT log2 copy number value. The cutoff point is set as the value yielding maximum sum of sensitivity and specificity for DFS analysis.

Figure 2

PCAT-1 facilitates the progression of breast cancer. a Expression of PCAT-1 in MCF-7, MDA-MB-231 and MCF10A cells was analyzed by qRT-PCR and normalized to GAPDH expression. b Expression of PCAT-1 in MCF-7 cells treated with different concentrations of cobalt chloride for 24 h or under hypoxic

conditions. MDA-MB-231 cells were cultured in normoxia or hypoxia. MTT assay showing growth (c), wound healing assay showing migration (d), transwell assay showing invasion (e) and colony formation (f) were detected in MDA-MB-231 cells transfected with PCAT-1 siRNA. Extracellular acid ratio (ECAR) level (g) and lactate production (h) in the culture media were determined after knockdown of PCAT-1 in MDA-MB-231. 2-DG, 2-deoxyglucose. The data are showed as fold difference compared with the level of negative control (NC) siRNA-transfected cells in normoxia. Data are shown as the mean±s.e.m. *P<0.05 and **P<0.01 compared with control.

Figure 3

Effects of PCAT-1 on HIF-1 α stability. a Effects of PCAT-1 overexpression on HIF-1 α protein expression in MCF-7 cells. b Effects of PCAT-1 overexpression on HIF-1 α mRNA expression in MCF-7 cells. Cells were cultured in normoxia or hypoxia for 1 h, and then the mRNA of HIF-1 α was examined by qRT-PCR and normalized to GAPDH expression. c Effects of PCAT-1 overexpression on HIF-1 α mRNA stability. MCF-7 cells were cultured in normoxia or hypoxia, then the cells were incubated with actinomycin D (5 μ g/ml) for 3 h, and the mRNA of HIF-1 α was examined at the indicated times by qRT-PCR. d Effects of PCAT-1 overexpression on HIF-1 α protein stability in the cells during reoxygenation. MCF-7 cells were cultured in hypoxia (1% O₂, 6h) and incubated for 1h in the presence of the proteasome inhibitor MG132 (10 μ mol/l) and cycloheximide (50 μ g/ ml) , then the cells were exposed to normoxia for the indicated times. e Effects of PCAT-1 knockdown on HIF-1 α protein expression in MDA-MB-231 cells under hypoxic conditions. f Effects of PCAT-1 knockdown on HIF-1 α mRNA expression and stability in MDA-MB-231 cells. g Effects of PCAT-1 knockdown on HIF-1 α protein stability in MDA-MB-231 cells during reoxygenation. Cells were cultured in hypoxia (1% O₂, 6h) and incubated for 1h in the presence of the proteasome inhibitor MG132 (10 μ mol/l) and cycloheximide (50 μ g/ ml), then the cells were exposed to normoxia for the indicated times. h Immunoblot analysis showed the nuclear translocation of HIF-1 α into the nucleus in MDA-MB-231 cells transfected with PCAT-1 siRNA in hypoxia. i Expression of HIF-1 α target genes in MDA-MB-231 cells transfected with PCAT-1 siRNA was measured by qRT-PCR. j Expression of HIF-1 α target genes in MCF-7 cells overexpressed PCAT-1 was measured by qRT-PCR under hypoxic conditions.

Figure 4

a PCAT-1-RACK1 Interaction Profile and PCAT-1-RACK1 Interaction Matrix. The interaction profile shows a peak at PCAT-1 Nucleotide Position 1200. The interaction matrix shows RACK1 Residue Index (0-300) vs PCAT-1 Nucleotide Index (0-1800).

b Co-immunoprecipitation of RACK1 and PCAT-1. Input, Antisense, and PCAT-1 are shown. RACK1 and Biotin-RNA are detected.

c Percent of input RACK1 in PCAT-1 IP. IgG and RACK1 are shown.

d RACK1 binding to PCAT-1 fragments. Input, 1-400, 401-800, 801-1299, and 1-1299 are shown. RACK1 binding is detected.

e RACK1 binding to PCAT-1 fragments. Input, PCAT-1 (1-800), and PCAT-1 (1-1299) are shown. RACK1 and Biotin-RNA are detected.

f HIF-1α expression. Vector, PCAT-1 (1-1299), and PCAT-1 (1-800) are shown. HIF-1α and β-actin are detected.

g Relative expression of HIF-1α target genes. Vector, PCAT-1 (1-1299), and PCAT-1 (1-800) are shown. VEGFA, ANGPTL4, CCND2, PDGF, PLOD2, LDHA, and GLUT1 are shown.

h Relative growth rate (%) under hypoxia. NC, si-PCAT-1#2, and si-PCAT-1#2+ΔPCAT-1(801-1299) are shown.

i Wound closure under hypoxia. NC, si-PCAT-1#2, and si-PCAT-1#2+ΔPCAT-1(801-1299) are shown. Wound closure (%) is shown.

j Cell invasion under hypoxia. NC, si-PCAT-1#2, and si-PCAT-1#2+ΔPCAT-1(801-1299) are shown. Cell invasion (%) is shown.

k Cell colony formation under hypoxia. NC, si-PCAT-1#2, and si-PCAT-1#2+ΔPCAT-1(801-1299) are shown. Number of colonies (%) is shown.

l RACK1 binding to GST-RACK1 fragments. RACK1 (WD1-WD7) is shown. Percent of binding is shown.

Figure 5

Bioinformatics prediction and biochemical identification of the interaction between PCAT-1 and RACK1. a CatRAPID fragments module prediction of the binding sites between PCAT-1 and RACK1. b The interaction between PCAT-1 and RACK1 was performed by RNA pull-down assay with a biotinylated PCAT-1 probe in MDA-MB-231 cells under hypoxic conditions. c RIP analysis of the interaction between PCAT-1 and RACK1 was performed using the anti-RACK1 antibody in MDA-MB-231 cells, and the level of PCAT-1 was determined using qRT-PCR. The truncated PCAT-1 were used in the RNA pull-down assay to identify the core regions of PCAT-1 for the physical interaction with RACK1 (d) and (e). f HIF-1 α stability and the expression of HIF-1 α target genes (g) were examined in MCF-7 cells overexpressed full-length PCAT-1 (nucleotides 1-1299) or truncated PCAT-1 (nucleotides 1-800) under hypoxic conditions. Endogenous PCAT-1 was depleted with siRNA in MDA-MB-231 cells, and the truncated PCAT-1 (nucleotides 801-1299) was overexpressed in the cells. The growth (h), migration (i), invasion (j) and colony formation (k) were analyzed in hypoxia. **P<0.01 compared with PCAT-1 knockdown group. l Schematic diagram of RACK1 functional domains are shown at the top. GST-tagged full-length or deletion mutants of RACK1 were transfected into MDA-MB-231 cells, and the potential interactions between PCAT-1 and the deletion mutants were detected by qRT-PCR under hypoxic conditions. Coomassie Blue staining of the GST fusion proteins is shown at the lower panel.

Figure 6

Effect of PCAT-1 on RACK1 binding to HIF-1α. a Co-immunoprecipitation of HIF-1α/RACK1 and HIF-1α/HSP90 interactions in MDA-MB-231 cells transfected with shRNA-PCAT-1. b Co-immunoprecipitation of HA-HIF-1α (P402A/P564A)/RACK1 and HA-HIF-1α (P402A/P564A)/HSP90 interactions in MCF-7 cells cotransfected with PCAT-1 and HA-HIF-1α (P402A/P564A) in the presence or absence of 17-AAG (0.5 mM) under hypoxic conditions. c RACK1 dimerization was detected in MDA-MB-231 cells cotransfected with shRNA-PCAT-1 and FLAG-RACK1 in hypoxia. d Serine phosphorylation of RACK1 was determined in MDA-MB-231 cells knockdown of PCAT-1.

Figure 7

PCAT-1 is required in cancer progression and metastasis. a Representative images of tumors in xenograft mouse models bearing MDA-MB-231 cells transfected with shRNA-Control or shRNA-PCAT-1. Tumors were harvested at 5 weeks after implantation. b The volume of tumor was measured every 5 days. c The weights of tumor were measured after 5 weeks when mice were sacrificed. The ratio of weight of lung tissue (d) and liver tissue (e) to weight of whole body reflected the metastasis ability of MDA-MB-231 cells after tail vein injection. Data are shown as mean±s.e.m. **P<0.01 compared with control.

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