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CUL4A Ubiquitin Ligase Is an Independent Predictor of Overall Survival in Pancreatic Adenocarcinoma

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Abstract. *Background/Aim: Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with dismal prognosis. Genomic instability due to defects in cell-cycle regulation/mitosis or deficient DNA-damage repair is a major driver of PDAC progression with clinical relevance. Deregulation of licensing of DNA replication leads to DNA damage and genomic instability, predisposing cells to malignant transformation. While overexpression of DNA replication-licensing factors has been reported in several human cancer types, their role in PDAC remains largely unknown. We aimed here to examine the expression and prognostic significance of the DNA replication-licensing factors chromatin licensing and DNA replication factor 1 (CDT1), cell-division cycle 6 (CDC6), minichromosome maintenance complex component 7 (MCM7) and also of the ubiquitin ligase regulator of CDT1, cullin 4A (CUL4A), in PDAC. Materials and Methods: Expression levels of CUL4, CDT1, CDC6 and MCM7 were evaluated by immunohistochemistry in 76 formalin-fixed paraffin-embedded specimens of PDAC patients in relation to DNA-damage response marker H2AX, clinicopathological parameters and survival. We also conducted bioinformatics analysis of data from online available databases to corroborate our findings. Results: CUL4A and DNA replication-licensing factors were overexpressed in patients with PDAC and expression of CDT1 positively correlated with H2AX. Expression of CUL4A and CDT1 positively correlated with lymph node metastasis. Importantly, elevated CUL4A expression was associated with reduced overall survival and was an independent indicator of poor prognosis on multivariate analysis. Conclusion: Our findings implicate CUL4A, CDT1, CDC6 and MCM7 in PDAC progression and identify CUL4A as an independent prognostic factor for this disease.*

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive, treatment-resistant malignancy and the third-leading cause of cancer-related death in the USA (1, 2). Despite recent advances in targeted therapies, treatment responses and survival rates in PDAC remain extremely low (3). Therefore, elucidation of molecular pathways involved in the progression of PDAC and identifying novel biomarkers that would inform prognosis and treatment decisions is crucial.

Point mutations and variations in chromosomal structure are major drivers in pancreatic carcinogenesis (4). Several proto-oncogenes and tumor-suppressor genes are critically involved in pancreatic carcinogenesis, with KRAS proto-oncogene, GTPase (*KRAS*), tumor protein p53 (*TP53*), SMAD family member 4 (*SMAD4*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and AT-rich interaction domain 1A (*ARID1A*) being the most commonly mutated genes that characterize PDAC (4-6). Genes amenable to targeted therapies such as Erb-b2 receptor tyrosine kinase 2 (*ERBB2*) and MET proto-oncogene, receptor tyrosine kinase (*MET*) also contribute to PDAC but with low prevalence (4). In recent years, genome-wide studies have unraveled the complex genomic landscape of pancreatic cancer, identifying a considerable proportion of tumors with genomic instability that is partially related to a defective DNA-damage response

(DDR) (4, 6). Importantly these 'unstable' genotypes show responsiveness to platinum-based therapy and DNA-targeting agents and therefore are clinically relevant for treatment selection for patients (6). Moreover, high replication stress, a feature commonly encountered in the squamous subtype of pancreatic cancer, represents another oncogenic pathway that is amenable to treatment with cell-cycle checkpoint inhibitors and shows great promise in terms of biomarker-driven treatment decisions (7).

Chromatin licensing for replication is an important regulatory mechanism that ensures genetic integrity (8). DNA replication-licensing occurs during the G_1 phase of the cell cycle and begins with the formation of multiprotein pre-replicative complexes at the origins of replication (9), consisting of the origin recognition complex (ORC) complex, cell division cycle 6 (CDC6), chromatin licensing and DNA replication factor 1 (CDT1) and minichromosome maintenance complex components 2-7 (MCM2-7). During the formation of pre-replicative complexes, CDT1 is responsible for the loading of MCM2-7 replicative helicase onto the origins (9-11). After origin firing, the pre-replicative complex is inactivated through different mechanisms to prevent re-firing of origins that would lead to replicative stress, DNA damage and genomic instability (9, 12). Regulation of CDT1 relies on the inhibitory protein geminin, and on ubiquitin-dependent proteolysis during the S phase (13-16). Three complexes with ubiquitin ligase activity are responsible for inhibiting CDT1 activity, namely SKP1-cullin-1-F-box protein containing SKP2 complex (SCF^{SKP2}), denticleless E3 ubiquitin protein ligase homolog CUL4–$DDB1^{CDT2}$ and the anaphase-promoting complex/cyclosome-CDH1 $(APC/C)^{CDH1}$ complex (13, 17). Several studies have shown that deregulation of DNA replication-licensing factors is significantly implicated in cancer (18-20). Overexpression of CDT1 has been reported to promote carcinogenesis through increased genomic instability (14, 21, 22). The ligase complex CUL4–$DDB1^{CDT2}$ also has an important role in carcinogenesis as it targets multiple regulators of the cell cycle and DDR (23), and new drugs that inhibit these ligases are promising anticancer agents.

Since the roles of CUL4A, CDT1, CDC6 and MCM7 in pancreatic cancer are largely unknown, the aim of the present study was to evaluate their expression in a series of PDACs in relation to DDR (shown by the marker H2AX) and prognosis.

Materials and Methods

Patients. Formalin-fixed paraffin-embedded (FFPE) PDAC tissues from a total of 76 patients were analyzed. Sixty (60) out of these 76 patients (78.94%) underwent pancreaticoduodenectomy (Whipple procedure), 4/76 (5.26%) total pancreatectomy and 10/65 (15.78%) distal pancreatectomy at the Department of Surgery, University Hospital of Patras, Greece from 2000 to 2020. FFPE PDAC tissue samples were retrieved from the Archives of the Department of Pathology, University Hospital of Patras. The study was approved by the University of Patras Ethics and Research Committee according to an institutional standardized protocol that abides by the Declaration of Helsinki (Approval Number 23453/09-10-2017). Forty-four patients (57.9%) were males and 32 (42.1%) were females, with a median age of 66 years (range=44-83 years). All tumors were graded according to the eighth edition of the tumor-node-metastasis staging system (24, 25). Patients who died postoperatively were excluded from the study. The median follow-up was 30.00 months (standard error of the mean (SEM)=3.48 months (range=2.00-156.00 months). The demographical, pathological and clinical information of the patients are presented in Table I.

Immunohistochemistry. Immunohistochemistry was performed on FFPE samples using a two-step immunoperoxidase method with diaminobenzidine as the chromogen (EnVisionTM FLEX Mini Kit High pH, K8023; DAKO, Carpinteria, CA, USA) as previously described (26). Primary antibodies and appropriate positive and negative controls used in the study are shown in Table II. Immunohistochemical staining was conducted and evaluated by an expert pathologist (VB) blinded to the case, using the weighted histoscore (H-score) according to the formula: (1×% cells staining weakly positive) + (2×% cells staining moderately positive) + (3×% cells staining strongly positive), resulting in scores ranging from 0-300 as previously described (26). Images were captured on a Nikon Eclipse 80i with ACT-1C software (Nikon Instruments Inc., New York, NY, USA).

Statistical analysis. Statistical analysis was conducted using SPSS statistical software (version 26.0; IBM, Armonk, NY, USA). Quantitative variables are expressed as the mean±SEM or as the median with interquartile range. Categorical variables are expressed as absolute and relative frequencies. Correlations between protein expressions (histoscores) were assessed with Spearman's correlation test. Differences between groups were tested with non-parametric tests (Kruskal-Wallis for more than two or the Mann-Whitney test for two independent samples). Statistical significance was set at $p<0.05$. For survival analysis, receiver operating characteristic curves were first plotted in order to categorize the expression levels of CUL4A, CDT1, CDC6, MCM7 and H2AX as high or low according to the optimal cutoff value of their respective histoscore. Life-table analyses were used to calculate cumulative survival rate with standard error (SE) for specific time intervals. Kaplan-Meier survival estimates were graphed over the follow-up period. The prognostic value of each variable was first assessed by univariate Cox regression analysis. Only variables that showed significant association with survival were included in the multivariate Cox proportional-hazard model in a stepwise method in order to determine the independent predictors for survival. The assumption of proportional hazards was evaluated by testing for interaction with a continuous time variable. Hazard ratios (HR) with 95% confidence intervals (95% CI) were computed from the Cox regression analyses. All reported *p*-values are two-tailed.

Bioinformatics analysis. Data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression projects (GTEx) were processed using Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/) (27). Association of CUL4A, CDC6, MCM7, H2AX and CDT1 with overall survival was investigated using the Kaplan–Meier method and the log-rank test.

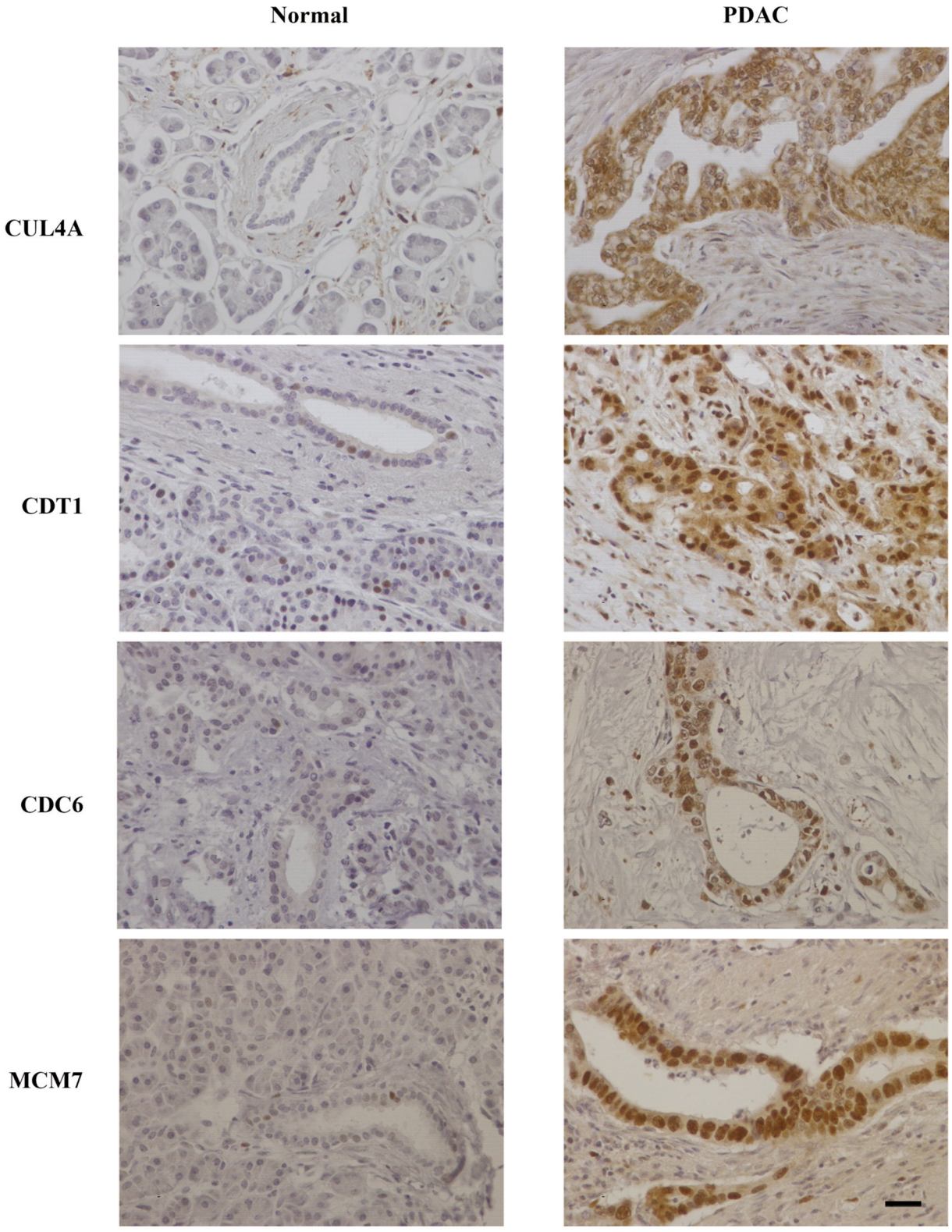

Figure 1. *Cullin 4A (CUL4A), chromatin licensing and DNA replication factor 1 (CDT1), cell division cycle 6 (CDC6) and minichromosome maintenance complex components 7 (MCM7) are overexpressed in human pancreatic ductal adenocarcinoma (PDAC). Representative cases showing strong immunohistochemical expression of CUL4A (B) and DNA replication-licensing factors CDT1 (D), CDC6 (F) and MCM7 (H) in PDAC compared to adjacent non-neoplastic pancreas. Magnification ×400. Scale bar=20 μm.*

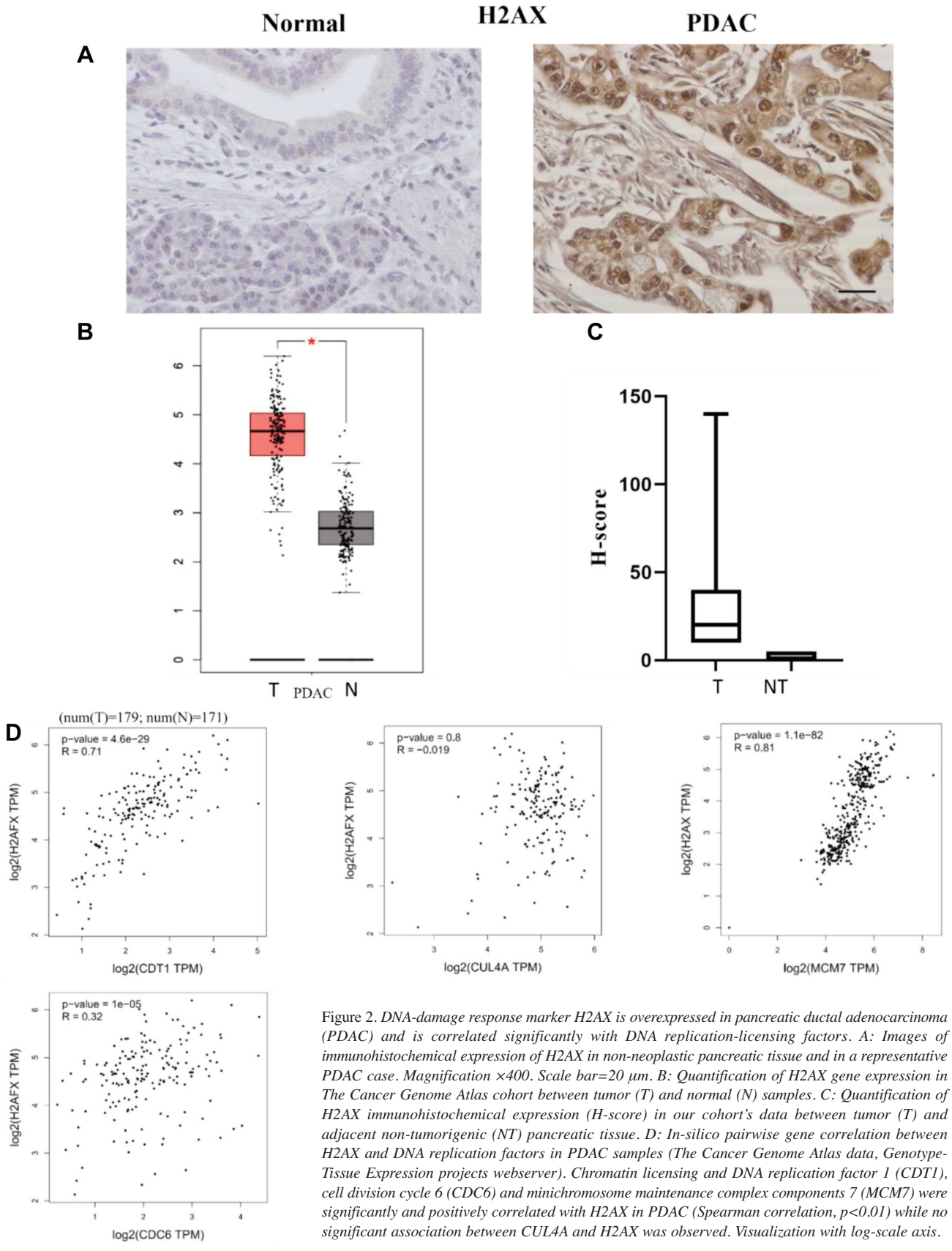

Figure 2. *DNA-damage response marker H2AX is overexpressed in pancreatic ductal adenocarcinoma (PDAC) and is correlated significantly with DNA replication-licensing factors. A: Images of immunohistochemical expression of H2AX in non-neoplastic pancreatic tissue and in a representative PDAC case. Magnification ×400. Scale bar=20 μm. B: Quantification of H2AX gene expression in The Cancer Genome Atlas cohort between tumor (T) and normal (N) samples. C: Quantification of H2AX immunohistochemical expression (H-score) in our cohort's data between tumor (T) and adjacent non-tumorigenic (NT) pancreatic tissue. D: In-silico pairwise gene correlation between H2AX and DNA replication factors in PDAC samples (The Cancer Genome Atlas data, Genotype-Tissue Expression projects webserver). Chromatin licensing and DNA replication factor 1 (CDT1), cell division cycle 6 (CDC6) and minichromosome maintenance complex components 7 (MCM7) were significantly and positively correlated with H2AX in PDAC (Spearman correlation, p<0.01) while no significant association between CUL4A and H2AX was observed. Visualization with log-scale axis.*

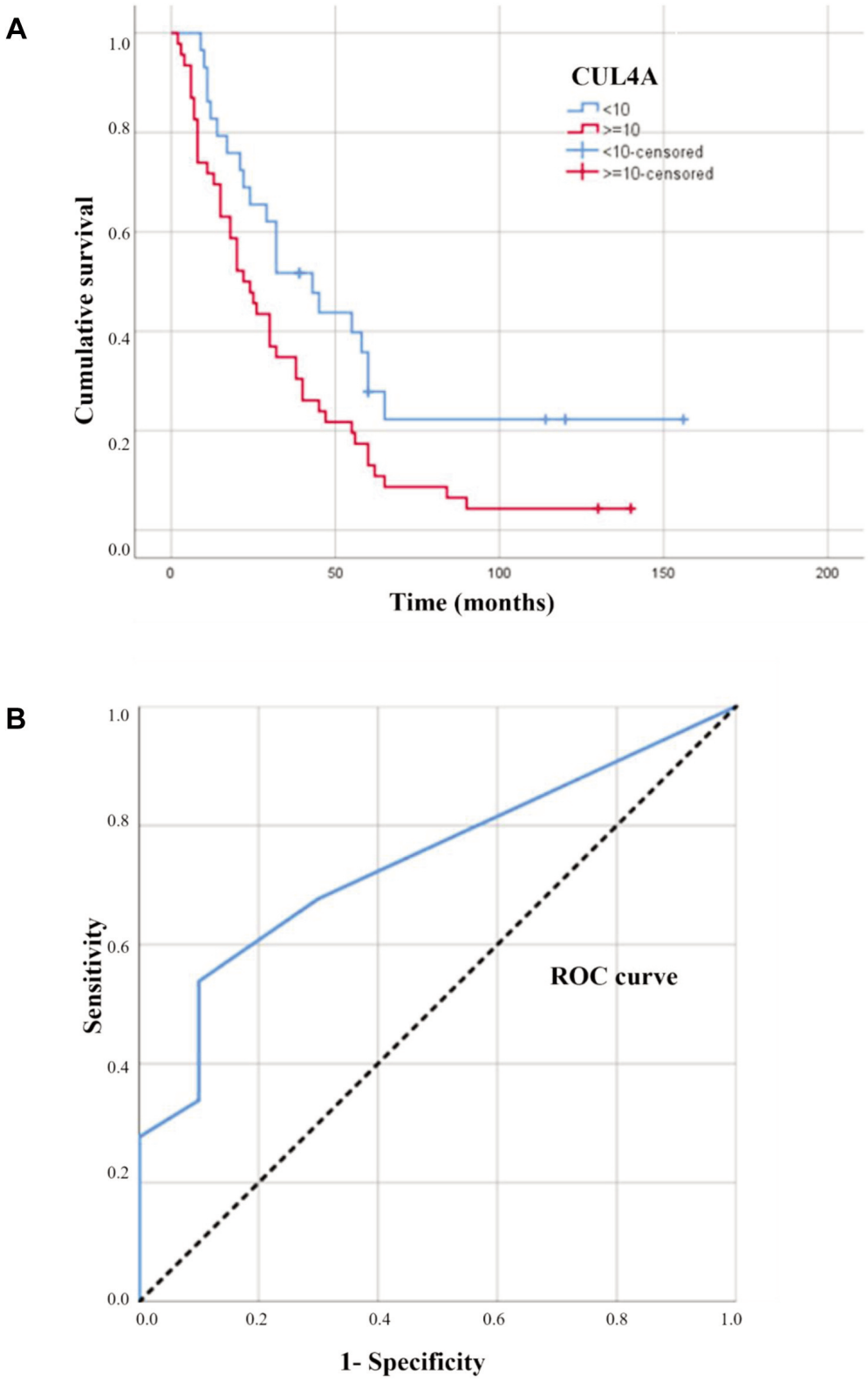

Figure 3. *Overall survival outcomes according to cullin 4A (CUL4A) expression in patients with pancreatic ductal adenocarcinoma. A: Kaplan–Meier survival estimates (p=0.012). B: Receiver operating characteristics (ROC) curve analysis for CUL4A. AUC is 0.74 (95% confidence interval=0.60-0.88) with p=0.01; sensitivity 67.7% and specificity 70% (hazard ratio=1.84, 95% confidence interval=1.09-3.10, p=0.022).*

Table I. *Demographical, pathological, and clinical information of the study patients (n=76).*

Characteristic		Value
Age, years	Mean±SD	66±9.6
Sex, n (%)	Male	44 (57.9)
	Female	32 (42.1)
Grade, n (%)	1	10 (13.9)
	2	39 (54.2)
	3	23 (31.9)
LN metastasis, n (%)	No	34 (44.7)
	Yes	42 (55.3)
Tumor size, n patients (%)	T1	13 (17.1)
	T2	23 (30.3)
	T3	38 (50)
	T4	2 (2.6)

LN: Lymph node; SD: standard deviation.

Log-rank values of $p<0.05$ were considered statistically significant. Expression of CUL4A, CDT1, CDC6, MCM7 and H2AX in PDAC *versus* normal (matched adjacent tissue from TCGA and GTEx data) was analyzed using GEPIA and RNA seq data. The expression data as transcript count per million (TPM) were transformed [log2(TPM+1] for differential analysis and the $\log_2$fold-change was defined as: median(Tumor)−median(Normal). Moreover, correlations between CUL4A, CDT1, CDC6 and MCM7 were examined in GEPIA, using Spearman correlation. For Spearman correlation, the genes were also log2(TPM) transformed.

Results

CUL4A and DNA replication-licensing factors CDT1, CDC6 and MCM7 are overexpressed in PDAC and are associated with parameters of tumor progression. We first examined expression of DNA replication-licensing factors by immunohistochemistry in our cohort of human PDAC samples. In adjacent non-neoplastic tissue, expression of all factors was negative or weakly positive in epithelial cells of pancreatic ducts or acini. In contrast, CUL4A, CDT1, CDC6 and MCM7 were overexpressed in cancer cells (Figure 1 and Supplementary Figure 1). Specifically, positive CUL4A immunohistochemical expression was found in 75/76 (98.7%) of the cases with a mean H-score of 82.7±6.8. CUL4A Immunoreactivity was localized in the nucleus or the cytoplasm of cancer cells in 48/76 cases (63.2%) and 73/76 cases (96.1%), respectively, with mean H-scores of 34.4±5.6 and 131.1±9.5, respectively. 61/76 (80.3%) cases of PDAC showed positive CDT1 expression, with a mean H-score 20.4±3.7, with nuclear and cytoplasmic localization in 62/76 cases (81.6%) and 10/76 (13.2%) cases, respectively (mean H-score 40.8±6.7 for nuclear and 0.7±0.6 for cytoplasmic). CDC6 and MC7 were also expressed in 72/76 (94.7%) cases (mean H-score of 36.4±3.9 and 41.1±3.9 respectively) with

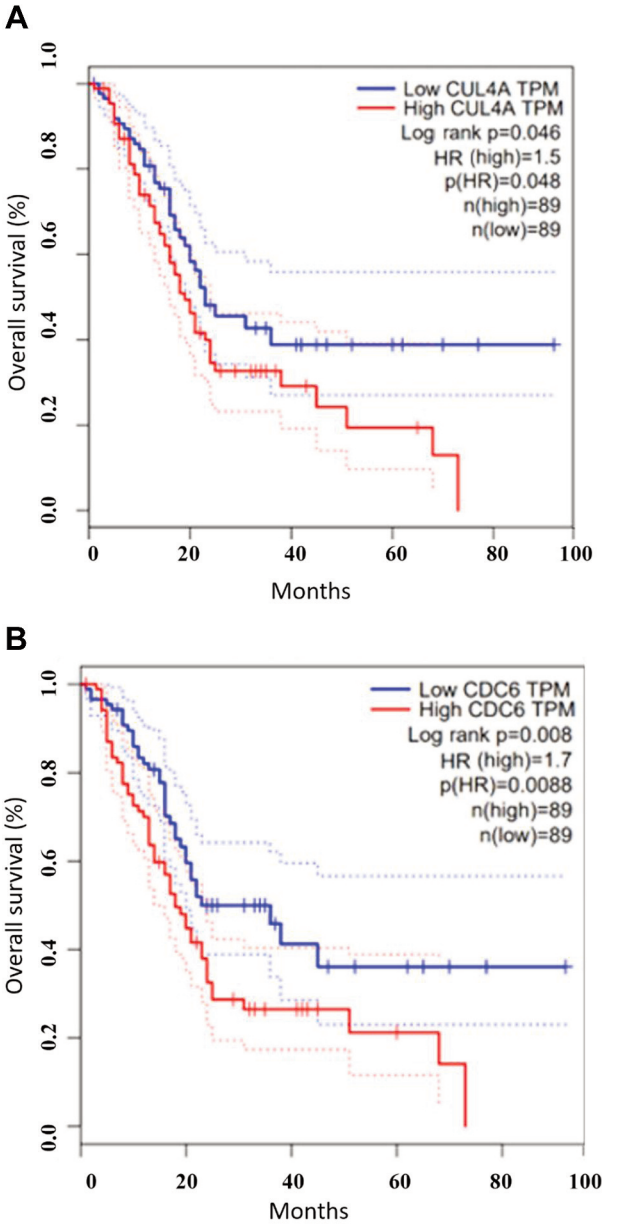

Figure 4. *Kaplan–Meier survival estimates according to expression of cullin 4A (CUL4A) (A) and cell division cycle 6 (CDC6) (B) in patients with pancreatic ductal adenocarcinoma. High CUL4A and CDC6 expression were associated with reduced overall survival (p=0.046 and p=0.008, respectively). Data from 178 patients were analyzed using log-rank tests based on gene expression from The Cancer Genome Atlas pancreatic ductal adenocarcinoma samples in the GEPIA webserver. Hazard ratios (HRs) are shown.*

nuclear and cytoplasmic localization. Nuclear and cytoplasmic CDC6 immunolocalization was observed in 68/76 cases (89.5%) and 32/76 (42.1%) cases with mean H-score 46.6±5.6 and 26.2±5.3 respectively, while nuclear and cytoplasmic

Table II. *List of antibodies used in the immunohistochemical study.*

Catalog #	Provider	Antibody	Antigen	Dilution/ application	Positive control	Negative control
PA5-49716	Thermo Fisher Scientific Inc., Waltham, MA, USA	Polyclonal	CUL4A	1:800	Colorectal carcinoma (55)	Rabbit immunoglobulin fraction (X0936; DAKO, Hamburg, Germany)
HPA003898	Sigma-Aldrich, St. Louis, MO, USA	Rabbit polyclonal	MCM7	1:80	Hepatocellular carcinoma (45)	
#PA5-29021	Thermo Fisher Scientific Inc. Waltham, MA, USA	Rabbit polyclonal	CDT1	1:100	Hepatocellular carcinoma (45)	
PA5-29167	Thermo Fisher Scientific Inc. Waltham, MA, USA	Rabbit polyclonal	CDC6	1:600	Colorectal carcinoma (48)	
#9718	Cell Signaling, Danvers, MA, USA	Rabbit monoclonal	p-H2AX (Ser139)	1:50	Hepatocellular carcinoma (45)	

Table III. *Outcome according to patient characteristics by univariate Cox regression analysis.*

		Death					
		No		Yes		HR (95% CI)	*p*-Value
Age	Mean (SD)	65.2 (10.1)		66.1 (9.5)		1.02 (0.99-1.05)	0.190
Sex	Males	5 (11.4)		39 (88.6)		Reference	
	Females	5 (15.6)		27 (84.4)		0.86 (0.52-1.40)	0.539
Grade	1	3 (30)		7 (70)		Reference	
	2	3 (7.7)		36 (92.3)		2.11 (0.93-4.81)	0.075
	3	3 (13)		20 (87)		2.01 (0.85-4.78)	0.114
LN metastasis	No	7 (20.6)		27 (79.4)		Reference	
	Yes	3 (7.1)		39 (92.9)		1.99 (1.21-3.27)	**0.006**
Tumor size	1	2 (15.4)		11 (84.6)		Reference	
	2	6 (26.1)		17 (73.9)		0.96 (0.45-2.06)	0.923
	3-4	2 (5.0)		38 (95.0)		1.66 (0.85-3.26)	0.141
H-Score		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)		
MCM7	Cytoplasmic	33 (26.8)	27.5 (10-60)	25.4 (38.5)	0 (0-40)	1.00 (0.93-1.09)	0.916
	Nuclear	70.5 (65.6)	55 (20-110)	53.3 (48.4)	47.5 (15-75)	0.99 (0.95-1.04)	0.793
CUL4A	Cytoplasmic	97 (64.5)	120 (30-130)	136.3 (83.5)	130 (60-190)	1.03 (0.99-1.06)	0.056
	Nuclear	6 (12.6)	0 (0-10)	38.8 (50.9)	20 (0-50)	1.16 (1.10-1.23)	**<0.001**
CDC6	Cytoplasmic	20 (27.9)	5 (0-60)	27.1 (47.7)	0 (0-30)	1.02 (0.95-1.08)	0.609
	Nuclear	33 (59.7)	12.5 (10-30)	48.7 (45.6)	30 (10-70)	1.01 (0.96-1.05)	0.796
H2AX	Cytoplasmic	13.3 (40)	0 (0-0)	0 (0)	0 (0-0)	0.77 (0.47-1.28)	0.316
	Nuclear	17.2 (24.8)	10 (5-20)	27.4 (31.6)	15 (10-40)	1.07 (0.99-1.15)	0.095
CDT1	Cytoplasmic	0 (0)	0 (0-0)	0.8 (5.3)	0 (0-0)	0.86 (0.54-1.35)	0.507
	Nuclear	61.9 (86.3)	17.5 (0-115)	37.3 (49.3)	20 (5-50)	0.98 (0.94-1.03)	0.481

CDC6: Cell-division cycle 6; CDT1: chromatin licensing and DNA replication factor 1; CI: confidence interval; CUL4A: cullin 4A; HR: hazard ratio; IQR: interquartile range; LN: lymph node; MCM7: minichromosome maintenance complex component 7; SD: standard deviation. Statistically significant *p*-values are shown in bold.

MCM7 immunoreactivity was detected in 67/76 cases (88.2%) and 39/76 (51.3%) cases with mean H-score 55.7±6.0 and 26.5±4.4, respectively. Overexpression of DNA replication-licensing factors in PDAC compared to normal pancreas was also shown by analyzing RNA seq data form TCGA and GTEx datasets using GEPIA (Supplementary Figure 1).

Nuclear immunohistochemical expression of CUL4A and CDT1 in our cohort was significantly higher in patients with lymph node metastasis (p=0.047 and p=0.02, respectively) than those without. Nuclear CUL4A expression was also positively associated significantly with tumor grade, with grade 2 tumors showing higher

expression of CUL4A compared to grade 1 (p=0.039) (Supplementary Figure 2).

There was a significant positive correlation between immunohistochemical expression of nuclear CUL4A and nuclear CDC6 in our cohort of PDAC samples (Spearman correlation R=0.3, p=0.011), while *in-silico* pairwise gene correlation using GEPIA also showed significant positive correlation between gene expression levels of *CUL4A*, *CDT1*, *CDC6* and *MCM7* in PDAC (Spearman correlation, p<0.01) (Supplementary Figure 3).

DDR marker H2AX is overexpressed in PDAC and is significantly correlated with DNA replication-licensing factors. Immunohistochemical expression of H2AX was observed in 63/76 (82.9%) PDAC cases, with a mean H-score of 13.9±2.3, while negative expression was found in adjacent non-neoplastic pancreatic tissue (Figure 2). Localization of H2AX was mainly nuclear in 63/76 (82.9%) cases, with a mean H-score of 26.1±3.7), while cytoplasmic immunoreactivity was found only in 6/76 (7.9%) cases (mean H-score of 1.7±1.6). There was a significant positive correlation between immunohistochemical expression of H2AX and CDT1 in our cohort (Spearman correlation R=0.64, p<0.0001), while no significant correlation was observed between H2AX and CUL4A, MCM7 or CDC6 expression (Supplementary Table II). However, *In-silico* pairwise gene correlation, using GEPIA and TCGA and GTEx datasets showed that *H2AX* gene expression correlated significantly with *CDT1*, *CDC6* and *MCM7* but not with *CUL4A* gene expression levels in PDAC (Spearman correlation, p<0.01) (Figure 2).

High CUL4A expression is a significant factor indicating poor prognosis in patients with PDAC. We next evaluated the prognostic significance of CUL4A, CDT1, CDC6, MCM7 and H2AX in PDAC. In our cohort, 65/74 (86.8%) patients with PDAC died during the follow-up period, with mean and medial survival times of 43.5 months (SE=5.1 months) and 30 months (SE=3.5 months), respectively. The overall survival curve for the entire cohort according to the Kaplan-Meier method is presented in Supplementary Figure 4. Life-table results are presented in Supplementary Table I. The probability of 1-, 2-, 5- and 10-year survival was 77.6% (SE=4.8%), 57.9% (SE=5.7%), 25.4% (SE=5.1%) and 10% (SE=3.7%), respectively. Outcomes according to patients' characteristics are presented in Table III. Kaplan-Meier analysis showed the statistically significant association of high nuclear CUL4A expression with reduced overall survival (p=0.012, Figure 3A).

Univariate survival analysis with Cox models revealed lymph-node metastasis and high nuclear CUL4A expression to be significant predictors of poor prognosis (HR=1.99, p=0.006 for lymph node metastasis; and HR=1.16, p<0.001 for high nuclear CUL4A). Further multivariate analysis indicated that CUL4A expression and lymph-node metastasis (HR=1.14, 95% CI=1.07-1.21, p<0.001; and HR=1.83, 95% CI=1.09-3.07, p=0.022, respectively) to be independent indicators of poor prognosis in PDAC The prognostic ability of CUL4A was examined *via* receiver operating characteristics analysis (Figure 3B). The area under the curve was 74% (95% CI=60-88) with p=0.015, indicating significant prognostic ability. The optimal cut-off for the CUL4A histoscore was ≥10, with a sensitivity of 68% and a specificity of 70%. Patients with a CUL4A histoscore ≥10 had 180% greater hazard (95% CI=1.09-3.1, p=0.022) than those with CUL4A <10.

We then performed bioinformatics analysis to validate our results. Survival analysis using GEPIA webserver revealed that high CUL4A and CDC6 expression were associated with reduced overall survival (p=0.046 and p=0.008, respectively). For MCM7, CDT1, and H2AX, no significant association with survival was observed (Figure 4).

Discussion

Identifying novel pathogenic mechanisms, and prognostic and predictive biomarkers in PDAC is crucial to selecting the most appropriate therapeutic approach and improving patient outcomes. An increasing body of evidence suggests that defective DDR mechanisms and replication stress constitute important mechanisms in PDAC progression, with potential clinical utility (4, 7). Herein, we report that overexpression of CUL4A and DNA replication-licensing factors are associated with tumor progression and DDR in PDAC and, importantly, we identify CUL4A as an independent predictor of unfavorable outcome.

CUL4A and DNA replication-licensing factors CDT1, CDC6 and MCM7 were overexpressed in our cohort of PDAC, findings that are further supported by our bioinformatics analysis of large datasets of PDAC. Moreover, high expression of CUL4A and CDT1 proteins in PDAC correlated with lymph node metastasis. To the best of our knowledge, this is the first study showing that CUL4A and DNA replication-licensing factors are implicated in tumor progression of PDAC in humans.

Our findings substantiate the known significant role of CUL4A in human carcinogenesis, emanating from its involvement in diverse functions, such as DDR and cell-cycle regulation (28). CUL4A targets degradation sensors of DNA damage and several cell-cycle regulators, including the DNA replication-licensing factor CDT1, thus preventing repeat replication of the genome during the S-phase of the cell cycle (28-34). *CUL4A* amplification or overexpression has been found in many human malignancies, including breast cancer (35), hepatocellular carcinoma (36, 37), lung cancer (38) and

colorectal cancer (39). Although CDT1 is a major target of CUL4A, correlation was not found between the two in our cohort of PDACs. Instead, we show the positive correlation of CUL4A with CDC6 in our case series, while CUL4A correlated with all the studied DNA-replication-licensing factors in PDAC by *in-silico* analysis of online available datasets. This is not surprising considering that CUL4A has a handful of targets regulating DDR and cell-cycle progression that may be involved in pancreatic carcinogenesis (28, 40). Nevertheless, additional studies and mouse models are required to gain a comprehensive understanding of the role of CRL4 in pancreatic cancer.

In further agreement with overexpression of DNA replication-licensing factors in human PDAC, deregulation of replication licensing has been shown to cause replication stress, DNA damage and genomic instability, contributing to malignant transformation (41-43). In this context, we previously showed CDT1 to be overexpressed in colorectal cancer (21, 44) and hepatocellular carcinoma (45), and several reports support the tumor-promoting roles of CDT1, CDC6 and MCM7 in other cancer types (45-50). Interestingly, CDC6 disruption in pancreatic cancer cells leads to chromosomal instability and it is potentially linked to the KRAS signaling pathway (47, 51, 52). In further agreement, a bioinformatics study based on TCGA data retrieved by Peng *et al*. supports the implication of MCM overexpression in PDAC progression (53). Another study in pancreatic neuroendocrine neoplasms also reported that MCM7 is a valuable marker for assessing tumor progression (54).

In line with data showing that deregulation of origin firing causes replication stress and DNA breaks, activating DDR (41-43), we report here that CDT1 expression correlated with DDR marker H2AX in our cohort, while H2AX correlated with all DNA replication-licensing factors in PDAC as shown by our bioinformatics analysis of TCGA and GTEx datasets. This is in agreement with results in human colorectal cancer showing that CDT1 overexpression causes origin over-licensing, activation of the DDR and increased genomic instability *in vivo*, thereby favoring cancer development (21). Given that replication stress was recently shown to characterize a subset of PDACs that are likely to respond to ATR serine/threonine kinase (ATR) and WEE1 G2 checkpoint kinase inhibitors, it would be very interesting to investigate whether overexpression of DNA replication-licensing factors in PDAC would represent biomarkers of clinical relevance or novel therapeutic targets.

An important novel finding of our study is that high CUL4A expression was an independent factor predicting poor prognosis in our cohort of PDACs, and this was further supported by bioinformatics analysis of large datasets. Although validation in future prospective studies is required, our results are in line with several studies reporting CUL4A to be associated with poor prognosis in other cancer types, including colorectal cancer (55), cholangiocarcinomas (56), non-small-cell lung cancer (38), breast cancer (57), and prostate cancer (58). Considering that several studies support the notion that CUL4A represents a promising candidate for therapeutic intervention, further evaluation of the significance of CUL4A in PDAC would be of considerable clinical value (59).

Conclusion

Our study provides novel evidence that CUL4A and DNA replication-licensing factors CDT1, CDC6 and MCMC7 are implicated in pancreatic cancer progression, possibly through mechanisms that involve replication stress and DNA damage, as shown by the positive correlation with DDR marker H2AX. Importantly, we show high CUL4A expression in PDAC is an independent predictor of poor survival. Further studies of chromatin-licensing deregulation in PDAC may identify biomarkers to help clinicians to accurately predict survival and guide treatment decisions.

Supplementary Material

Supplementary material available at:
https://doi.org/10.6084/m9.figshare.25043759

Conflicts of Interest

The Authors do not have any disclosures to report.

Authors' Contributions

TP: Data curation, formal analysis, investigation, methodology, software, validation, visualization, and writing – original draft. NS: Investigation, methodology, and visualization. GC: Investigation. BP. Data curation, CTSC Investigation and software. MM: Data curation and validation. MI: Funding acquisition, investigation, methodology, project administration, resources, software, supervision, and validation. BV: Conceptualization, methodology, project administration, resources, software, supervision, validation, visualization, and writing – review and editing.

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