Protein Arginine Deiminase enzymes which citrullinate epitopes for MHC II presentation are independent predictors of survival in colorectal cancer

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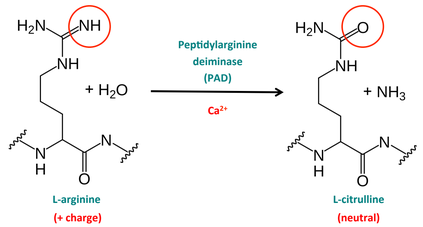
Introduction

- Protein Arginine Deiminases (PADs) are a family of Ca^{2+} dependent enzymes that are activated under cellular stress within the tumor environment.
- PADs citrullinate protein substrates to generate modified self-antigens.



Citrullination. A modification that occurs within stressed cells. Peptidylarginine deiminase (PADs) enzymes are activated and converts arginine to citrulline by altering the positively charge aldimine group (=NH) group of arginine to the neutrally charged ketone group (=O) of citrulline.

- T cells targeting modified self-antigens play a role in the pathophysiology of several autoimmune diseases.
- Presentation of citrullinated peptides on MHC class II stimulates CD4 T cells to mediate potent anti-tumor immunity (1)
- In this study we focus on the role of the PadI2 and PadI4 members in colorectal cancer.

Methods

- Using a colorectal TMA and immunohistochemistry, expression of PadI2 and PadI4 was assessed.
- Tumors were classified a H-Score and assessed for strong, moderate, weak and negative PadI2 and PadI4 expression.
- All statistical analysis was performed using SPSS21 software.
- A study cohort (n=462) of tumor samples were analysed to determine their association with clinicopathological variables, other tumor markers and the effect on survival.

PadI2 and PadI4 Expression in Colorectal tissue

Number of cores stained for PadI2 and expression levels

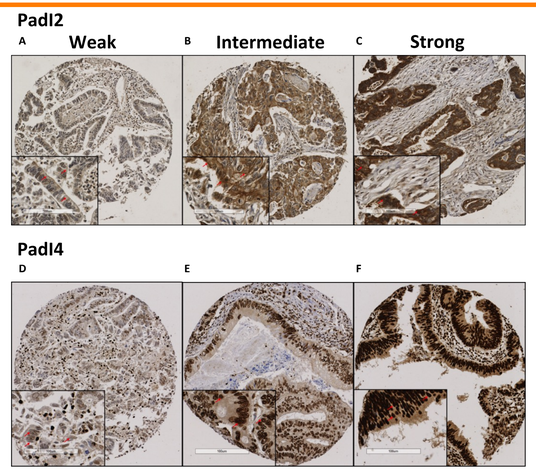
Expression of PadI2		Frequency	Percent	Valid Percent
	Negative	18	3.9	6.2
	Weak	153	33.1	52.5
Valid	Moderate	102	22.1	35.1
	Strong	18	3.9	6.2
	Total	**291**	63.0	100.0
Missing	System	171	37.0	
Total		462	100.0	

PadI2 expression was mainly detected in the cytoplasm. Out of 462 tumors, 291 were stained with a monoclonal antibody directed against PadI2 (clone pAB0197). 37% of cases could not be evaluated due to absence of enough tissue or no evaluable tumor cells (i.e. stroma) in the core. Only 6.2% failed to stain, 52.5% weakly, 35.1% moderate and 6.2% strongly.

Number of cores stained for PadI4 with cytoplasmic PadI4 expression levels

Expression of Cytoplasmic PadI4		Cytoplasmic		
		Frequency	Percent	Valid Percent
	Negative	0	0	0
	Weak	63	13.6	27.3
Valid	Moderate	143	31.0	61.9
	Strong	25	5.4	10.8
	Total	**231**	50.0	100.0
Missing	System	231	50.0	
Total		462	100.0	

Out of 462 tumors, 231 were stained with a monoclonal antibody directed against PadI4 (clone pAB0199). 50% of cases could not be evaluated. All cases stained strongly for PadI4 within the nucleus (not shown in table). In the cytoplasm 27.3% stained weakly, 61.9%moderate and 10.8% strongly.



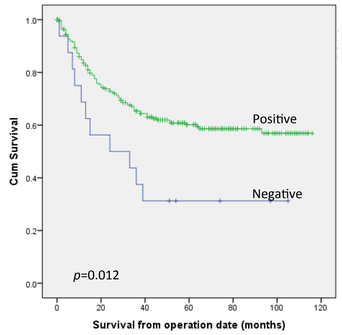
Photomicrographs of colorectal TMA cores immunohistochemically stained for PadI2 (A-C) and PadI4 (D-F). The level of expression ranged from (A-D) weak, (B-E) Intermediate and (C-F) strong. All X10 magnification (insets X20). Arrows point to areas representative of cytoplasmic and nuclear staining in PadI2 and PadI4 cores respectively.

Correlation of PadI2 and PadI4 Expression With standard clinicopathological variables

Variable	No.of cases Total cohort (%), n = 462	Frequency of the PadI2 stained cohort (%), n =291		Frequency of the PadI4 stained cohort (%), n=231		
		Stained cohort %	χ2 test (p Value)	Stained cohort %	χ2 test (p Value) Nuclear	χ2 test (p Value) Cytoplasm
Gender						
Male	266 (57.6)	164 (56.4)	0.435	136 (58.9)	0.108	0.148
Female	196 (42.4)	127 (43.6)		95 (41.1)		
Histologic tumor type						
Adenocarcinoma	392 (84.8)	250 (85.9)		196 (84.8)		
Mucinous	51 (11)	30 (10.3)		26 (11.3)		
Columnar	4 (0.9)	1 (0.3)	0.283	2 (0.9)	0.008*	0.597
Signet ring	7 (1.5)	4 (1.4)		3 (1.3)		
Unknown	8 (1.7)	6 (2.1)		4 (1.7)		
Tumor grade (differentiation)						
Well	29 (6.3)	21 (7.2)		15 (6.5)		
Moderate	353 (76.4)	212 (72.9)	0.751	169 (73.2)	0.128	0.570
Poor	71 (15.4)	51 (17.5)		43 (18.6)		
Unknown	9 (1.9)	7 (2.4)		4 (1.7)		
Tumor site						
Colon	238 (51.5)	153 (52.6)		119 (51.5)		
Rectal	181 (39.2)	111 (38.1)	0.124	87 (37.7)	0.603	0.535
Unknown	43 (9.3)	27 (9.3)		25 (10.8)		
TMN stage						
0	3 (0.7)	1 (0.3)		1 (0.4)		
I	69 (14.9)	38 (13.1)		29 (12.6)		
II	174 (37.7)	108 (37.1)	0.910	85 (37.1)	0.297	0.205
III	155 (33.5)	107 (36.8)		84 (36.4)		
IV	54 (11.7)	32 (11.0)		27 (11.7)		
Unknown	7 (1.5)	5 (1.7)		5 (2.2)		
Vascular Invasion status						
Negative	224 (48.5)	144 (49.5)		110 (47.6)		
Positive	128 (27.7)	79 (27.1)	0.439	61 (26.4)	0.888	0.283
Missing	110 (23.8)	68 (23.4)		60 (26.0)		

Univariate analysis was performed to determine whether PadI2/PadI4 expression correlated with standard clinicopathological variables. Pearsons χ^2 and Fishers exact test indicated that PadI2 did not correlate with any of the variables whereas nuclear but not cytoplasmic PadI4 showed a strong association with histological type (p=0.008).

PadI2 Expression increases patient overall survival



Kaplan-Meier survival analysis indicates a correlation with PadI2 expression and survival (log rank test, p=0.012).

Expression	Mean survival time (Months) in relation to PadI2 expression				p Value
	Estimate (Months)	Std. Error	95% Confidence Interval Lower Bound	Upper Bound	
Negative	44.813	10.483	24.267	65.358	
Positive	76.181	3.184	69.941	82.422	0.012
Overall	74.391	3.104	68.307	80.475	

Expression of PadI2 increased survival from 44.81 months (95% CI 24.27 to 65.36) to 76.18 months (95% CI 69.94 to 82.42)

Correlation of PadI2 Expression with other markers

Variable	χ2 test (p Value)
ENO-1 Nuclear	0.213
ENO-1 Cytoplasm	0.103
Bcl2	0.404
Distant Mets	0.875
MicA	0.234
P53	0.278
Ki-67	0.046*
MHC class I combined	0.815
Stat1 Nuclear	0.220
Stat1 Cytoplasmic	0.066
Microsatellite Instability	0.233
MHC class II in tumour	0.573
ITCC	0.340
Vimentin	0.660
PadI4 Cytoplasmic	0.063
PadI4 Nuclear	0.392

Univariate analysis indicated that PadI2 did not show any significant association with the cell cycle/survival regulators p53, BCL2, nuclear STAT1 as well as the immune markers MHC class I and II involved in immunosurveillance/ editing.

No significant correlation was observed with the cytoskeletal protein Vimentin or the glycolytic enzyme Alpha Enolase (ENO-1) both known substrates that are citrullinated by Pad enzymes.

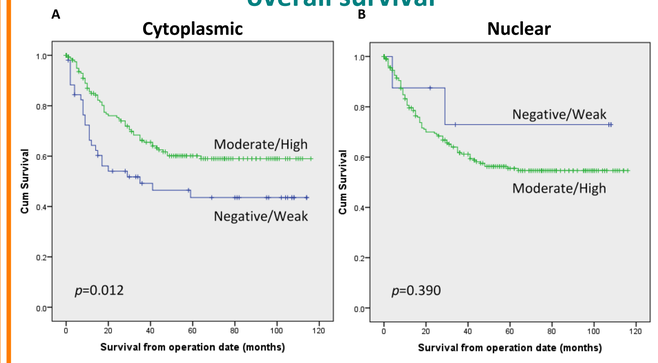
PadI2 expression only significantly associated with expression of the Nuclear antigen Ki67 (p=0.046), a cellular marker for proliferation.

PadI2 Expression is a independent marker of good prognosis in colorectal cancer

Variable	Category	HR (ExpB)	95% CI	p Value
TNM Stage	I and II	1	0.218 to 0.653	0.000
	III and IV	0.378		
Vascular Invasion	Negative	1	0.239 to 0.626	0.000
	Positive	0.387		
PadI2	Negative	1	1.034 to 3.918	0.040
	Positive	2.013		
MicA	Negative/Weak	1	1.196 to 2.878	0.006
	Moderate/High	1.855		

Multivariate analysis using Cox regression showed that expression of PadI2 and the stress related protein MicA, TMN stage and Vascular Invasion all have independent prognostic significance.

Cytoplasmic PadI4 Expression increases patient overall survival



In Kaplan-Meier survival analysis tumors expressing moderate/high levels of cytoplasmic but not nuclear PadI4 were associated with improved survival (log rank test, p=0.012).

Expression	Mean survival time (Months) in relation to PadI4 expression				p Value
	Estimate (Months)	Std. Error	95% Confidence Interval Lower Bound	Upper Bound	
Negative/weak	57.993	7.325	43.635	72.350	
Moderate/high	77.321	3.944	69.590	85.051	0.012
Overall	72.691	3.543	65.747	79.634	

Expression of cytoplasmic PadI4 increased survival time from 57.99 months (95% CI 43.64 to 72.35) to 77.32 months (95% CI 69.59 to 85.05)

Correlation of PadI4 Expression with other markers

Variable	χ2 test (p Value)	
	Nuclear	Cytoplasmic
ENO-1 Nuclear	0.345	<0.001*
ENO-1 Cytoplasm	0.001*	<0.001*
Bcl2	0.067	0.028*
Distant Mets	0.372	0.049*
MicA	0.390	0.086
P53	0.609	0.478
Ki-67	0.380	0.190
MHC class I combined	0.213	0.257
Stat1 Nuclear	0.115	0.289
Stat1 Cytoplasmic	0.696	0.333
Microsatellite Instability	0.555	0.544
MHC class II in tumour	0.360	0.388
ITCC	0.613	0.202
PadI2 (pad2hnp)	0.392	0.063
Vimentin	0.469	0.450
PadI4 Cytoplasmic	<0.001*	N/A
PadI4 Nuclear	N/A	<0.001*

Univariate analysis showed that expression of Nuclear PadI4 is significantly associated with only the cytoplasmic enzyme Alpha Enolase (p=0.001).

In contrast Cytoplasmic PadI4 is highly significantly associated with Alpha Enolase located in both the cytoplasm (p=0.001) and the shorter nuclear form (p=0.001) known as MBP-1, a transcription factor that downregulates the activity of the c-Myc proto-oncogene acting as a tumor suppressor.

Cytoplasmic PadI4 expression also significantly correlated with expression of the anti- apoptotic protein BCL2 (p=0.028).

Cytoplasmic PadI4 Expression is a independent marker of good prognosis in colorectal cancer

Variable	Category	HR (ExpB)	95% CI	p Value
TNM Stage	I and II	1	0.168 to 0.582	0.000
	III and IV	0.313		
Vascular Invasion	Negative	1	0.341 to 0.974	0.040
	Positive	0.577		
PadI4 (Cytoplasmic)	Negative/Weak	1	1.040 to 3.068	0.036
	Moderate/High	1.786		
MicA	Negative/Weak	1	1.089 to 3.032	0.022
	Moderate/High	1.817		

Multivariate analysis using Cox regression identified cytoplasmic PadI4, TMN stage, Vascular Invasion and the stress related protein MicA as independent markers of good prognosis.

Conclusion

Patients with tumor that expresses either PadI2 or Cytoplasmic PadI4 have a better prognosis in colorectal cancer.
After multivariate analysis both remained independent prognostic factors.
Results are consistent with the hypothesis that Pads are activated within stressed tumor cells leading to generation and presentation of citrullinated epitopes that are recognised by T cells and killed. Tumor growth is controlled by T cells and the patient has a better prognosis.

(1) Brentville VA, Metheringham RL, Gunn B, Symonds P, Daniels I, Gijon M, Cook K, Xue W, Durrant LG (2016). Cancer Research 2016 Feb 1;76(3):548-60