Downregulation of the Deiminase PADI2 Is an Early Event in Colorectal Carcinogenesis and Indicates Poor Prognosis

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Abstract

Peptidyl arginine deiminases (PADI) are a family of enzymes that catalyze the poorly understood posttranslational modification converting arginine residues into citrullines. In this study, the role of PADIs in the pathogenesis of colorectal cancer was investigated. Specifically, RNA expression was analyzed and its association with survival in a cohort of 98 colorectal cancer patient specimens with matched adjacent mucosa and 50 controls from donors without cancer. Key results were validated in an independent collection of tumors with matched adjacent mucosa by mining of a publicly available expression data set. Protein expression was analyzed by immunoblotting for cell lines or IHC for patient specimens that further included 24 cases of adenocarcinoma with adjacent dysplasia and 11 cases of active ulcerative colitis. The data indicate that PADI2 is the dominantly expressed PADI enzyme in colon mucosa and is upregulated during differentiation. PADI2 expression is low or absent in colorectal cancer. Frequently, this occurs already at the stage of low-grade dysplasia. Mucosal PADI2 expression is also low in ulcerative colitis. The expression level of PADI2 in tumor and adjacent mucosa correlates with differential survival: low levels associate with poor prognosis.

Implications: Downregulation of PADI2 is an early event in the pathogenesis of colorectal cancer associated with poor prognosis and points toward a possible role of citrullination in modulating tumor cells and their microenvironment.

Introduction

Peptidyl arginine deiminases (PADI) are a family of enzymes that catalyze the conversion of arginine residues to citrullines (reviewed in ref. 1). Five genes located in a well-organized cluster on human chromosome 1 encode the five enzymes PADI1, PADI2, PADI3, PADI4, and PADI6. Of these, PADI2 is the ancestral homologue and widely expressed in different tissues, while the other PADI show restricted tissue-specific expression (summarized in ref. 2). Although PADI enzymes share a high degree of homology, they have some substrate specificity in vitro (3, 4). The citrullination reaction catalyzed by PADI enzymes is strictly calcium-dependent and effectively removes the positive charge of arginine residues. As such, this posttranslational modification has a profound influence on protein–protein interactions when occurring on relevant interaction surfaces. Methylation is another posttranslational modification of arginine residues. It has been shown that monomethylated but not dimethylated arginines can be citrullinated (5). Citrullination prevents posterior methylation (6). Important functions for PADI enzymes have been reported in both the cytosol and the nucleus. PADIs citrullinate components of cytoskeleton in the cytosol such as vimentin (7) but also proteins that have structural extracellular functions such as fibrin (8). A major function of PADI activity in the nucleus is decondensation of chromatin by citrullination of histone proteins (9).

This function has been suggested to contribute to the naive chromatin state of pluripotent embryonic stem cells (10). Through citrullination of histones and other chromatin regulators, PADIs have further been implicated in gene regulation (summarized in ref. 11).

In the innate immune response, chromatin decondensation upon histone hypercitrullination allows the formation of neutrophil extracellular trap (NET) formation that neutrophils use to capture bacteria (9). PADI enzymes are further involved in a range of auto-inflammatory diseases, including rheumatoid arthritis and multiple sclerosis due to their activation during NETosis, apoptosis, and autophagy (12). A common denominator in these diseases is an increase in enzymatic PADI activity and increased levels of antibodies against citrullinated antigens (1). These autoantigenic antibodies are used as biomarkers for diagnosis and prognosis for rheumatoid arthritis (13).

More recently, PADI enzymes, in particular PADI2 and PADI4, have been further implicated in cancer (11). PADI4 was found to be overexpressed in epithelial cells from various adenocarcinoma...
comparatively upregulated in human HER2-positive breast cancers (15). In mice, overexpression of a PADI2 transgene was sufficient to cause skin tumors (16). It has been proposed that PADIs could contribute to cancer by acting as coactivator of oncogenic transcription factors eliciting tumor-promoting expression programs. To illustrate this, PADI4 was shown to enhance transcriptional regulation of Tal1 in hematopoietic stem cells (17). However, the outcome of such a coactivator function is strongly dependent on the cellular context. In the estrogen receptor–positive subgroup of breast cancer patients, PADI2 expression correlated with good prognosis and in breast cancer cells was shown to facilitate the binding of the estrogen receptor to its target genes (18).

Colorectal cancer is the second most common cancer in the western world (19). The role of PADIs in the pathogenesis of colorectal cancer has not been investigated. In this article, we report that PADI2 is the primarily expressed PADI in colon mucosa and that its expression is low or absent in tumor tissue at an early stage of the colorectal cancer development. Moreover, higher level of PADI2 in the tumor and adjacent mucosa is a predictor of good prognosis.

Materials and Methods

Patient samples and data sets

The following sample series and data sets were analyzed: RNA from 16 colorectal tumors and matched normal mucosa; immunohistochemistry of tissue blocks from 24 patients with infiltrating adenocarcinoma containing areas of both low- and high-grade dysplasia and normal mucosa; immunohistochemistry of colon tissue blocks from 11 patients with active ulcerative colitis; microarray-based expression data from tumor and adjacent mucosa samples of a cohort of 98 stage II primary colon cancer patients and 50 colon mucosa samples from donors without cancer (ref. 20; www.colonomics.org). The project was approved by the Hospital’s Ethics Committee with registration number PR074/11. Samples were obtained after written, informed consent following the guideline of the Declaration of Helsinki. Also, RNA-seq–based expression data from The Cancer Genome Atlas (TCGA) were used for validation studies (21).

Cell culture

Colorectal cancer cell lines HCT116, HT-29, SW480, DLD-1, and Colo205 were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies; 11960085) supplemented with 10% FBS (Life Technologies; 10270106), 2 mmol/L glutamine, 1 mmol/L pyruvate, and 1% penicillin–streptomycin (Life Technologies; 25030024; 11360039; 15140122). CaCo2 cells were grown in DMEM–F12 (Life Technologies; 21331020) supplemented with 20% FBS, 2 mmol/L glutamine, 1 mmol/L pyruvate, and 1% penicillin–streptomycin. All cells were propagated with a split ratio of 1:10 every 2 to 3 days by trypsinization (0.05% Trypsin with EDTA 4Na 1x; Life Technologies, 25300062) and plating onto 10-cm² dishes. To differentiate Caco-2 cells, these were grown until they reached confluency onto 10-cm² dishes. Upon confluency, medium was changed every 2 days and cells were harvested after 20 days.

RT-qPCR and immunoblotting

Cell lysis, Western blot, and RNA extraction were performed as previously described (22). For immunoblotting, we have used antibodies directed against PADI2 (Proteintech UK; 12110-1-AP) and C-terminal histone H3 (Abcam; ab204964). RNA levels were quantified by RT-qPCR and normalized to two reference genes (HPRT1 and GAPDH). All oligo sequences are available on request.

Immunohistochemistry

Immunohistochemistry and hematoxylin–eosin staining on formalin-fixed paraffin-embedded tissue sections were performed as described (23). For immunohistochemistry, we have optimized the signal background for anti-PADI2 (Proteintech UK; reference number 12110-1-AP) using positive and negative control samples. A pathologist scored the expression of PADI2 as positive, focally positive, or negative.

Data analysis

To analyze TCGA data, we have used the Wanderer application that we had developed for this purpose and that is described in detail elsewhere (24). The analysis of expression microarray data for specific genes of interest was performed essentially as previously described (20). The comparison of expression between tumor samples (n = 98) and normal mucosa (n = 50) was performed with a linear model, adjusted for age and gender. The sample size for this discovery phase allowed to identify and effect size of 0.5 with 80% power. For the validation, the TCGA dataset had a similar power, and the series of matched paired samples (n = 16) could detect an effect size of 1.0 or larger. For the analysis of prognosis, disease-free survival curves were estimated using the Kaplan–Meier method for a categorization of PADI2 expression. Also, a multivariate Cox model was used to estimate hazard ratios, 95% confidence intervals and calculate P values from likelihood ratio tests. Analyses were adjusted for age and gender, but not for stage, because all cases were stage II colon cancer.

Results

PADI2 expression is downregulated in colorectal cancer

To investigate the expression of PADI family members we have analyzed RNA expression data from tumor and adjacent mucosa samples of 98 colon cancer patient cohort and 50 colon mucosa samples from donors without cancer (20). As shown in Fig. 1A, of all PADI genes, PADI2 is the only gene prominently expressed in colon mucosa. We found a pronounced and highly significant downregulation of PADI2 in tumor samples from the same patients. We found that PADI2 was downregulated in 15 of these 16 samples (Fig. 1B). In most samples the downregulation was more than 8-fold. Next, we analyzed available RNA-seq data from the TCGA Consortium. For this we used the Wanderer application that we had developed to facilitate the analysis of the rather bulky TCGA data for individual genes or loci (24). The data set based on 41 normal mucosa samples and 262 colorectal
adenocarcinomas further confirmed the downregulation of PADI2 with a Wilcoxon P value = 8.0e−23 (Fig. 1C). As promoter DNA methylation frequently correlates with the reduced gene expression (25), we have analyzed DNA methylation data from TCGA. However, we found no correlation of DNA methylation and PADI2 expression (data not shown). Taken together, these results show that downregulation of PADI2 is a frequent feature of colorectal cancer.

**PADI2 expression correlates with enterocytic differentiation**

Next, we analyzed the protein levels of PADI2 in a panel of established colorectal cancer cells lines, including many that are regularly used for molecular research, such as HCT116, HT-29, and SW480. PADI2 was virtually undetectable by immunoblotting in most cell lines (Fig. 2A). The only colorectal cancer cell line found to express low to moderate levels of PADI2 was HT-29. The MBU-TS4 cell line derived from transformed keratinocytes PADI2 has been included as a reference for high endogenous PADI2 expression (26). Our preliminary results suggest that overexpression of PADI2 in colorectal cancer cell lines at a comparable or even higher level than in MBU-TS4 cells does not affect the adhesion to collagen I (Supplementary Fig. S1A and S1B), but even higher level than in MBU-TS4 cells does not affect the adhesion to collagen I (Supplementary Fig. S1A and S1B), but might render HT-29 cells sensitive to calcium influx-induced reduction in proliferation and viability correlating with the transcriptional activation of the cell cycle inhibitory CDKN1A/p21 (Supplementary Fig. S1C and S1D).

Next, we addressed the question whether the low or absent expression of PADI2 could be a consequence of the dedifferentiated state of these colorectal cancer cell lines. For this we used CaCo2 cells that can be induced to differentiate in cells with mature enterocytic features (27). While undifferentiated CaCo2 cells were negative for PADI2 by immunoblotting, differentiated cells the protein was readily detected (Fig. 2B). Similarly, mRNA levels were strongly upregulated in differentiated CaCo2 cells (Fig. 2C). 

Immunohistochemical staining of normal colon mucosa further confirmed that expression of PADI2 is characteristic of mucosal enterocytes in the colon crypt (Fig. 2D). A similar expression of PADI2 was previously described in other epithelial cells from kidney, endometrium, and mammary gland (28).

**Downregulation of PADI2 expression is an early event in colonic tumourigenesis**

Colorectal cancer is a disease with a step-wise progression from early dysplasia to advanced adenocarcinoma with infiltrating features (29). To get an understanding at which step the expression of PADI2 is lost, we have performed anti-PADI2 immunohistochemistry in a set of samples from 24 colorectal cancer patients that contained normal mucosa, areas of dysplasia, and infiltrating adenocarcinoma in the same tissue sample (Fig. 3A). While PADI2 was expressed in normal mucosa, expression was frequently already lost in low-grade dysplasia. In 12 of 24 samples such dysplastic areas were negative for PADI2 and in another 7 samples expression was reduced and only focally retained (Fig. 3B). In line with the mRNA expression results of Fig. 1, PADI2 protein was not detected in the large majority (21 of 24) of infiltrating adenocarcinomas (Fig. 3A and B). These results argue in favor of PADI2 downregulation as an early event in colorectal tumourigenesis.

**PADI2 expression is also low in active ulcerative colitis**

In some cases, it is challenging to distinguish early low-grade dysplasia of colorectal cancer from reactive but noncancerous epithelia. This situation occurs in the setting of active ulcerative colitis, which is a form of inflammatory bowel disease. Ulcerative colitis is characterized by chronic and episodic colonic inflammation that affects adolescents and young adults with an etiology and pathogenesis that have not been fully elucidated (30). Histologically the process involves the mucosa at the anal portion and extends proximally in a contiguous fashion. The affected mucosa shows a lymphoplasmacytic inflammation in the lamina...
propia with cryptitis and crypt abscesses by neutrophilic infiltration (31). The repeated episodes of active inflammation increase the risk of colorectal cancer usually preceded by dysplasia (32). The identification of dysplasia in these patients is crucial to warrant the best management at a curable stage of their disease. However, biomarkers that allow distinguishing reactive but non-
cancerous epithelial alterations from dysplasia are not available yet. For this reason, we have analyzed the expression of PADI2 in 11 cases of active ulcerative colitis (Fig. 4). In all 11 cases, the expression of PADI2 was not detectable in the epithelial crypt cells arguing that the downregulation of PADI2 is not a specific

Figure 2. PADI2 expression is mostly absent in colorectal cancer cell lines but induced during enterocytic differentiation. A, crude cell lysates from colorectal cancer cell lines were analyzed by immunoblotting using anti-PADI2 and anti-Histone H3 antibodies. A lysate from MBU-TS4 squamous cell carcinoma cells known to express PADI2 were included as a positive control. B, crude cell lysates from proliferating and differentiated Caco-2 cells were analyzed by immunoblotting as in A. C, the level of PADI2 mRNA was quantified by RT-qPCR in proliferating and differentiated Caco-2 cells (n = 3; error bars = ± SD). D, immunohistological analysis of healthy colon mucosa using anti-PADI2 antibody.

Figure 3. Downregulation of PADI2 is an early event in the development of colorectal cancer. A, tissue samples containing areas of normal mucosa, low-grade dysplasia, and infiltrating adenocarcinoma were analyzed by anti-PADI2 immunohistochemistry and hematoxylin and eosin staining (n = 24). A representative example is shown. B, the expression of PADI2 was scored as positive, focally positive, or negative. A quantification of all 24 analyzed samples is shown.
PADI2 Is Lost in Colorectal Cancer

Lower levels of PADI2 in tumor and adjacent mucosa correlate with poor prognosis

Next, we took advantage of the availability of up to 10 years of follow-up information on the cohort of 98 colorectal cancer patients. We have used the expression range calculated for Fig. 1A to group patients according to their expression of PADI2. Adjacent mucosa and tumor samples required different cutoff points because the expression values had little overlap. For the tumor samples, the difference between the two groups was statistical significant at cutoff 5.4 (HR = 0.29; 95% CI, 0.09–0.98, P = 0.046). The tumor-adjacent mucosa required a higher expression level cutoff at 7.7, which was significantly associated with disease-free survival (HR = 0.82; 95% CI, 0.12–0.76, P = 0.011). Patients with lower PADI2 levels, either in tumor or adjacent mucosa had the worse prognosis (Fig. 5). It is well accepted that microenvironment can affect tumor growth and progression (33). Our results suggest that in addition to a possible tumor-intrinsic function, the absence of PADI2 in adjacent mucosa in colon cancer patients could be part of an altered tumor-promoting environment. Whether this aberrant loss is also present in some cancer-free individuals (Fig. 1A) conferring them higher cancer risk remains to be further studied.

Because PADI2 was also downregulated in noncancerous ulcerative colitis (Fig. 4), we wondered whether the association of PADI2 expression in adjacent mucosa with reduced survival could be explained tumor-surrounding inflammation. To address this question, we have estimated an inflammatory index using the expression data for 200 genes (listed in Table S1) extracted from the inflammatory hallmark gene set from database MSigDB (34). This signature has been derived from diverse studies of genes related to inflammatory response. Also, as indirect evidence of immune cell infiltration, we have used the expression of CD8A that has been described as a specific marker of T lymphocyte infiltration (35). The analysis of these two signatures correlated with PADI2 expression in adjacent normal mucosa, but in opposite directions. The inflammatory index showed an inverse correlation with PADI2, while CD8A was positively correlated (Supplementary Fig. S2A). In any case, the analysis of the prognostic value of PADI2 in adjacent normal mucosa remained significant (HR = 0.22; 95% CI, 0.06–0.81, Cox adjusted P = 0.023) after adjustment for age, gender, tumor site, hallmark inflammatory index, and CD8A expression. We have further used the R-package "estimate" (36) to infer an immune score that seemed unrelated to PADI2 expression (Supplementary Fig. S2A). Again the prognostic value of PADI2 expression remained significant when adjusting for immune score in addition to age, gender, tumor site, and inflammatory index (Supplementary Fig. S2B). Our results further suggest that PADI2 expression in adjacent mucosa has prognostic value independent of inflammation and immune cell infiltration.

Taken together, our results show that the absence or loss of PADI2 expression is a frequent and early event in colorectal cancerogenesis. Assessing the expression level of PADI2 in tumor or adjacent mucosa has a prognostic value for colorectal cancer. Our results further suggest that for tumor-adjacent mucosa this prognostic value is independent of inflammation and immune cell infiltration.
Discussion

PADI2 expression correlates with differentiation

Here, we report the downregulation of the peptidyl deiminase family member PADI2 at an early stage of colorectal cancer formation. The function of PADIs is best understood in skin. Keratinocytes express PADI isoforms 1–3 which citrullinate keratin 1 and filaggrin during the terminal differentiation of keratinocytes (37). The consequential accumulation of citrullinated proteins at the outer layers of the epidermis is thought to be a key event to maintain the cutaneous barrier function. Similar to epidermis, the colon mucosa is another example of an epithelium with a high cell turnover. Here, we show that PADI2 protein is present in enterocytes and, analogous to the epidermis, is predominantly expressed in the more differentiated cells at the tips of the villi and less in the crypts where the stem cell compartment is located (Fig. 2D). In this line, the mRNA encoding PADI2 was detected upregulated in a study comparing the global gene expression pattern of colon villi tops with crypts (38). While many colorectal cancer cells express very little PADI2, its expression is induced when forcing tumor cells to differentiate in vitro. Hence, we hypothesize that the apparent loss of PADI2 expression in colorectal cancer may be a consequence of reflecting the undifferentiated or dedifferentiated status of tumor cells.

Does PADI2 serve as early diagnosis biomarker?

At present, there is no biomarker to help pathologists distinguish between dysplastic changes and noncancerous lesions such as inflammation, thus choosing the most appropriate treatment scheme is challenging. Because reduction of PADI2 expression is an early event in colorectal cancer, PADI2 was tested as a biomarker. However, when analyzing PADI2 expression in active ulcerative colitis samples, we found that PADI2 is also downregulated in this noncancerous pathology. At first sight, this finding was somewhat surprising taking into account previous studies which have highlighted the role of PADI activity, in particular PADI4 in inflammatory processes (reviewed in ref. 39). In the context of ulcerative colitis, the pan-PADI inhibitor C1-amidine was found to be effective in a mouse model where it reduced inflammation (40). As an explanation, the authors found that the drug acted primarily on inflammatory immune cells and induced their apoptosis while epithelial cells remained unaffected (40). Another study reported increased levels of citrullination in inflammatory bowel disease (41). From their immunohistochemistry, it is apparent that a fraction of the citrullinated proteins are extracellular. As such, it would be interesting to determine to which extent infiltrating immune cells cause the observed citrullination and to what level epithelial cells contribute to it. Similarly, we lack any knowledge about the identity of the PADI enzyme responsible for the citrullination. It seems likely that PADI2 may have distinct roles in inflammatory cells and in differentiated epithelia. While the function of PADI2 and PADI4 in immune cells has been well described (7, 42, 43), the role of PADI2 in colonic epithelium remains unexplored. We now contribute with a novel result showing that the apparent loss of PADI2 expression occurs early in tumorigenesis. It remains to be determined if it can be used as a biomarker for colorectal cancer risk as a similar loss in epithelial expression also occurs in ulcerative colitis, which is a cancer predisposing condition.

PADI2 has an ambivalent role in cancer

Adding to the complexity, the role of PADI in tumors seems to be context dependent. In contrast to colorectal cancer, an oncogenic role for PADI2 has been reported in breast cancer and squamous carcinomas (15, 16). In a cell culture model for breast cancer progression, PADI2 expression correlated with the transformed phenotype and cells and xenografted tumors were sensitive to C1-amidine treatment (15). Mice overexpressing PADI2 in a panel of tissues including skin and mammary epithelia spontaneously developed squamous cell carcinomas in the skin in 37% of the cases but no other tumors (16). In vitro data suggested that overexpression of PADI2 in skin carcinomas could favor epithelial-to-mesenchymal transitions and thus increase the cells’ migratory capacity (16). In contrast, in colorectal cancer, PADI2 is downregulated and reduced expression in both tumor tissue and adjacent mucosa correlated with reduced survival. Reexpression of PADI2 in colorectal cancer cell lines had no influence on adhesion. Our preliminary experiments however suggested that PADI2 overexpression could render HT-29 cells sensitive to calcium treatment. Calcium treatment led to a reduction of viability and proliferation that correlated with the upregulation of the cell-cycle inhibitory gene CDKN1A/p21 (Supplementary Fig. S1C and S1D). This link between PADI2 and p21 is particularly interesting given that HT-29 cells harbor a change-of-function mutation in p53 and thus lack the classic p21-activating pathway. These observations warrant further exploration as any alternative pathway that leads to the activation of p21 might have therapeutic potential for the many p53 mutant cancers. Taken together, in colon cancer PADI2 seems to act as tumor suppressor, which contrasts with reported functions in skin and mammary cancer. The dual role of cancer genes as oncogenes and tumor suppressors is common, if not universal, in cancer pathogenesis and depends on the tissue context (44–46).

Why does PADI2 in adjacent mucosa predict survival?

Patients with higher PADI2 expression in the tumor or the adjacent mucosa have best prognosis and, conversely, downregulation of PADI2 correlates with poor survival. It is intriguing that the expression level of PADI2 in the peritumoral mucosa is associated with survival. As a possible explanation, it can be speculated that in addition to a tumor-intrinsic function, PADI2 might modulate the microenvironment in a way that when lost favors tumor progression. The modification of secreted components by PADI2 has the potential to provide a molecular mechanism. PADIs catalyze the conversion of charged arginine residues into neutral citrullines. The net removal of a positive charge has great potential to change the substrate’s protein–protein interaction capacity and function. In fact, Struyf and colleagues already demonstrated in vitro that citrullination of the cytokine CXCL12, expressed by colonic epithelial cells, reduces its binding affinity to CXCR4 and CXCR7 receptors located on monocyte and lymphocyte cell surfaces (47). As a result, PADI may play an anti-inflammatory role, attenuating the recruitment of CXCR4-positive leukocytes to sites of inflammation. Our finding that the predictive potential of PADI2 was independent of gene signatures related to inflammation and immune cell infiltration, however, argues against this. From studies in skin, PADIs are known to modify components of the extracellular matrix (37). Changes in the extracellular matrix have clear potential to affect adhesion and migration of embedded cancer cells. In a first exploratory set of experiments, however, we were unable to detect any influence of
PAD2 overexpression on the capacity of colorectal cancer cells to adhere to collagen. In a theory first put forward for oral carcinoma, it has further been suggested that changes in normal epithelium can facilitate tumor initiation by providing a permissive field for cancerization (48). In this context, hypermethylation and silencing of ADAMTS14, a member of the extracellular matrix metalloproteinases, in normal colon mucosa associates with a field effect for cancerization (49). Whether PAD2 could be part of such a field effect remains speculative at this stage.

Taken together, the lack of PAD2 expression is an early event in the development of colorectal cancer and its expression level correlates with differential survival. Future work will be required to determine the substrates and citrullination events that are relevant for this association and whether these alterations could have an application as biomarkers or points for therapeutic intervention.

Disclosure of Potential Conflicts of Interest

M.A. Peinado is Chief Scientific Officer at Aniling. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: N. Cantariño, J. Douet, M. Buschbeck

Development of methodology: N. Cantariño, V. Valero, J. Douet

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Cantariño, E. Musulén, V. Valero, M. Penacho, V. Moreno, M. Buschbeck

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