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Original Article

VNN1 overexpression is associated with poor response to preoperative chemoradiotherapy and adverse prognosis in patients with rectal cancers

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Abstract: Background: Colorectal cancer is prevalent worldwide and it is also the fourth most common cause of cancer mortality. For rectal cancer, neoadjuvant concurrent chemoradiotherapy (CCRT) followed by radical proctectomy is gold standard treatment for patients with stage II/III rectal cancer. By data mining a public dataset of rectal cancer transcriptome (GSE35452) from Gene Expression Omnibus, National Center of Biotechnology Information (GEO, NCBI), we identified that VNN1 was the most significantly upregulated gene among those related to nitrogen compound metabolic process (GO:0006807). Therefore, we analyzed the clinicopathological correlation and prognostic impact of VNN1 protein (pantetheinase), which encoded by VNN1 gene. Methods: VNN1 immunostaining was performed in 172 rectal adenocarcinomas treated with preoperative CCRT followed by surgery, which were bisected into high- and low-expression subgroups. Furthermore, statistical analyses were performed to correlate the relationship between VNN1 immunoreactivity and clinicopathological features, as well as three survival indices: disease-specific survival (DSS), local recurrence-free survival (LRFS) and metastasis-free survival (MeFS). Results: High VNN1 immunoexpression was significantly associated with advanced pre-treatment and post-treatment disease and poor response to CCRT (all $P \leq .026$). In addition, VNN1 overexpression was linked to adverse DSS, LRFS and MeFS in univariate analysis and served as an independent prognosticator indicating worse DSS and LRFS in multivariate analysis (all $P \leq .019$). Conclusion: VNN1 may play a crucial role in rectal cancer progression and responsiveness to CCRT, and serve as a novel prognostic biomarker. Additional studies to clarify the molecular pathway are essential for developing potential VNN1-targeted therapies for rectal cancer.

Keywords: CCRT, chemoradiotherapy, pantetheinase, rectal cancer, VNN1

Introduction

Colorectal adenocarcinoma is worldwide prevalent. It is ranked as the third most common cancer in men and the second in women, especially in the more developed countries [1]. The incidence also became much higher in Chinese population recently [2]. Preoperative chemoradiotherapy have been shown to have prognostic benefit for patients with rectal cancer of cT3/cT4 or nodal metastasis (stage II/III) [3, 4]. Nevertheless, the five-year survival rate is still unsatisfactory for all stages of colon and rectal cancer (65%) [5]. Accordingly, research and development for more effective therapeutic regimens are necessary for these patients.

Malignant tumors feature rapid growth and demand for large amount of energy and nutrients. To support viability of tumor cells and enlarge tumor biomass size in relatively nutrient-poor microenvironment, genetic or epigenetic alterations of cellular metabolism is important for oncogenesis. Nitrogen is one of the essential nutriments for organisms. Increased demand for nitrogen is also one of the hallmarks of cancer metabolism [6]. Via data mining of a Gene Expression Omnibus (GEO, National Center for Biotechnology Information (NCBI), Bethesda, MD, USA) transcriptomic database (GSE35452) with analysis of genes associated with nitrogen compound metabolic process (GO:0006807), we identified *VNN1* as

the most significantly upregulated gene in non-responders compared with responders.

VNN1 gene encodes pantetheinase, human analog of mouse vanin-1, which degrades pantetheine into pantothenic acid (vitamin B_5) and cysteamine [7]. *VNN1* gene expression level was significantly higher in blood-based samples of patients with colorectal cancer than controls [8, 9]. To the best of our knowledge, however, no study investigates the relationship between VNN1 expression and response to chemoradiotherapy or survival rates. Therefore, we conducted this research.

Materials and methods

Data mining of transcriptomic database from GEO to identify the most up-regulated gene

A transcriptomic dataset (GSE35452), comprising 46 cases of rectal cancer treated with preoperative chemoradiotherapy from GEO, NCBI, was selected for research. The tumors were dichotomized into "responder" and "non-responder" according to the response to preoperative chemoradiotherapy. In this dataset, GeneChip® Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, USA) was used for analysis. After downloading the raw CEL file, Nexus Expression 3 software (BioDiscovery, El Segundo, CA, USA) was used to perform comparative analysis without filtering or preselection. Under supervision, we inspected the statistical significance of each transcript by comparing responder and nonresponder, targeting the genes associated with nitrogen compound metabolic process (GO:0006807). Those genes with *P*-value < .01 and expression fold change > ± .1 $\log_2$ ratio were picked out for further evaluation.

Study cohort of patients and specimens

The Institutional Review Board of E-DA Hospital approved the study. Our study cohort comprised 172 patients with primary adenocarcinoma of rectum between 1998 and 2004. All the patients received preoperative chemoradiotherapy followed by radical proctectomy. The preliminary clinical staging was determined by endoscopic ultrasound (EUS) and abdominopelvic computed tomography (CT). Patients with distant metastasis at initial diagnosis, screened by chest plain film and abdominopelvic CT, were excluded. The detailed patient selection and the protocol of treatment were the same as previously described [10].

Histopathological assessment, immunohistochemical study and evaluation of VNN1 expression

Post-treatment staging was based on pathological examination of radical proctectomy according to 7th edition of American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system [11]. The grading system of tumor regression after chemoradiotherapy treatment was evaluated in accordance with the description of Dworak *et al.* [12]. For immunohistochemical study, tissue sections of pre-treatment tumor biopsy specimens were subjected to the routine procedures of deparaffinization, rehydration, and epitope retrieval. Subsequently, the tissue sections were proceeded to incubation with primary antibody against VNN1 (1:100, rabbit polyclonal, Cat No. ab205912, Abcam, Cambridge, United Kingdom) for one hour. Splenic tissues with or without incubation of VNN1 antibody were run in parallel as positive and negative control, respectively. We evaluated the expression of VNN1 protein by combination of the intensity and percentage of immunoreactivity in the tumor cells to produce an H-score. The equation for the H-score is as follows: H-score = $\sum$Pi(i + 1), in which Pi represents the percentage of stained tumor cells (0%-100%) and i represents the intensity of immunostaining (0-3+).

Statistical analyses

We used SPSS V.14.0 software package (SPSS Inc., Chicago, IL, USA) for statistical analysis. The VNN1 immunoreactivity median H-score was applied as the cutoff point to bisect out patient cohort into two groups, over- and under-expression. Using Pearson's χ^2 test, we compared the relationships between VNN1 expression and miscellaneous important clinical and pathological features. Three prognostic indices, disease-specific survival (DSS), local (pelvic) recurrence-free survival (LRFS) and metastasis-free survival (MeFS) intervals, were determined from the day of surgical excision to the day patient death caused by tumor, locoregional relapse of tumor, and occurrence of distant metastatic disease, respectively. For uni-

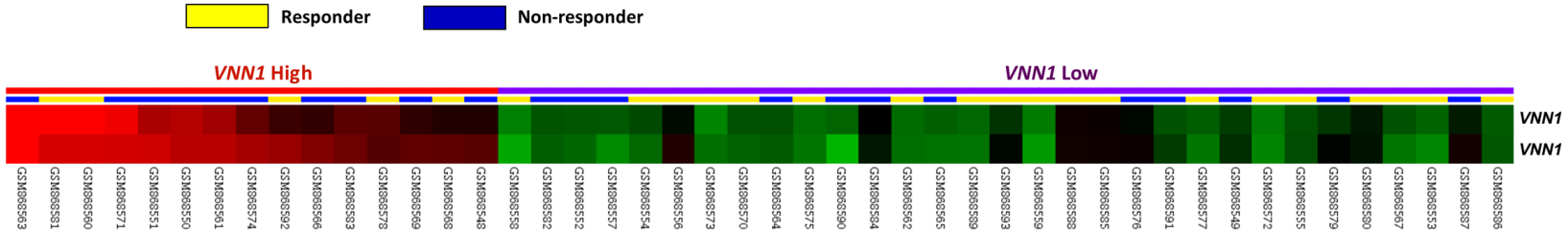

Figure 1. Analysis of gene expression in rectal cancers with neoadjuvant CCRT by using a published transcriptome dataset (GSE35452). Conducting a clustering analysis of genes by focusing on nitrogen compound metabolic process (GO:0006807) revealed that *VNN1* was the most significantly upregulated genes in non-responder comparing with responder. Tissue specimens from cancers classified as responder or non-responder are illustrated at the top of the heat map, and the upregulation and downregulation of gene expression are represented as a continuum of brightness of red or green, respectively. Specimens with unchanged transcriptional level are in black.

Table 1. Summary of differentially expressed genes associated with nitrogen compound metabolic process (GO:0006807) in relation to response to CCRT in rectal carcinoma

Probe	Comparison log ratio	Comparison *P*-value	Gene Symbol	Gene Name	Biological Process	Molecular Function
1558549_s_at	0.6644	0.0022	*VNN1*	Vanin 1	Cell motility, nitrogen compound metabolic process	GPI anchor binding, hydrolase activity, hydrolase activity; acting on carbon-nitrogen (but not peptide) bonds, hydrolase activity; acting on carbon-nitrogen (but not peptide) bonds; in linear amides
205844_at	1.2511	0.0002	*VNN1*	Vanin 1	Cell motility, nitrogen compound metabolic process	GPI anchor binding, hydrolase activity, hydrolase activity; acting on carbon-nitrogen (but not peptide) bonds, hydrolase activity; acting on carbon-nitrogen (but not peptide) bonds; in linear amides

Table 2. Associations and comparisons between VNN1 expression and clinicopathological factors in 172 rectal cancer patients receiving neoadjuvant CCRT

Parameter		No.	VNN1 Expression		*P*-value
			Low Exp.	High Exp.	
Gender	Male	108	55	53	0.752
	Female	64	31	33	
Age	< 70	106	51	55	0.531
	≥ 70	66	35	31	
Pre-Tx tumor status (Pre-T)	cT1-cT2	81	52	29	< 0.001*
	cT3-cT4	91	34	57	
Pre-Tx nodal status (Pre-N)	cN0	125	69	56	0.026*
	cN1-cN2	47	17	30	
Post-Tx tumor status (Post-T)	ypT1-ypT2	86	56	30	< 0.001*
	ypT3-ypT4	86	30	56	
Post-Tx nodal status (Post-N)	ypN0	123	65	58	0.237
	ypN1-ypN2	49	21	28	
Vascular invasion	Absent	157	82	75	0.059
	Present	15	4	11	
Perineurial invasion	Absent	167	85	82	0.173
	Present	5	1	4	
Tumor regression grade	Grade 0-1	37	12	25	0.001*
	Grade 2~3	118	59	59	
	Grade 4	17	15	2	

*, statistically significant.

variate survival analyses, Kaplan-Meier survival curves with comparison by log-rank test were used. Those parameters showing statistical significance in univariate analyses were enrolled in multivariate tests by using Cox proportional hazards model (Cox model). For all analyses, only *P* value less than .05 was judged as statistically significant under two-tailed tests.

Results

VNN1 recognized as the most significantly upregulated gene among those belonging to nitrogen compound metabolic process (GO:0006807)

In the public transcriptomic database of rectal cancer (GSE35452) from GEO, 24 out of 46 patients showed response to preoperative chemoradiotherapy (responder), the rest 22 patients belonged to non-responder. Twenty probes covering 13 transcripts associated with nitrogen compound metabolic process (GO:0006807) were found. Of these, only two probes covering *VNN1* transcript demonstrated significant up-regulation in non-responders than in responders (**Figure 1**). The $\log_2$ ratios by comparison between non-responders and responders were 0.6644- and 1.2511-fold up-regulation ($P \leq .0022$, **Table 1**).

Clinical and pathological features of patients with rectal adenocarcinoma

As shown in **Table 2**, the majority was male (n = 108, 62.8%) and less than 70 years old (n = 106, 61.6%) in our patient cohort of rectal cancer. About the pre-treatment (Pre-Tx) tumor staging, the invasive depth of 47.1% tumors (n = 81) were up to limited to muscularis propria (cT1-2), and 52.9% (n = 91) were beyond the muscularis propria (cT3-4); 27.3% (n = 47) had nodal metastasis, and 72.7% (n = 125) didn't. About the post-treatment (Post-Tx) tumor staging, the invasive depth of 50.0% tumors (n = 86) were up to limited to muscularis propria (ypT0-2), and 50.0% (n = 86) were beyond the muscularis propria (ypT3-4); 28.5% (n = 49) had nodal metastasis, and 71.5% (n = 123) didn't. Vascular invasion and perineural invasion were detected in 8.7% (n = 15) and 2.9% (n = 5) tumors, respectively. The response to preoperative chemoradiotherapy varied from grade 0-1 (n = 37, 21.5%), grade 2-3 (n = 118, 68.6%) and grade 4 (n = 17, 9.9%).

Association between VNN1 immunoreactivity and clinical and pathological variables

After dividing the patient cohort into VNN1 over- and under-expression subgroups with cutoff point of median H-score, we used Pearson's χ^2 test to compare the relationship between

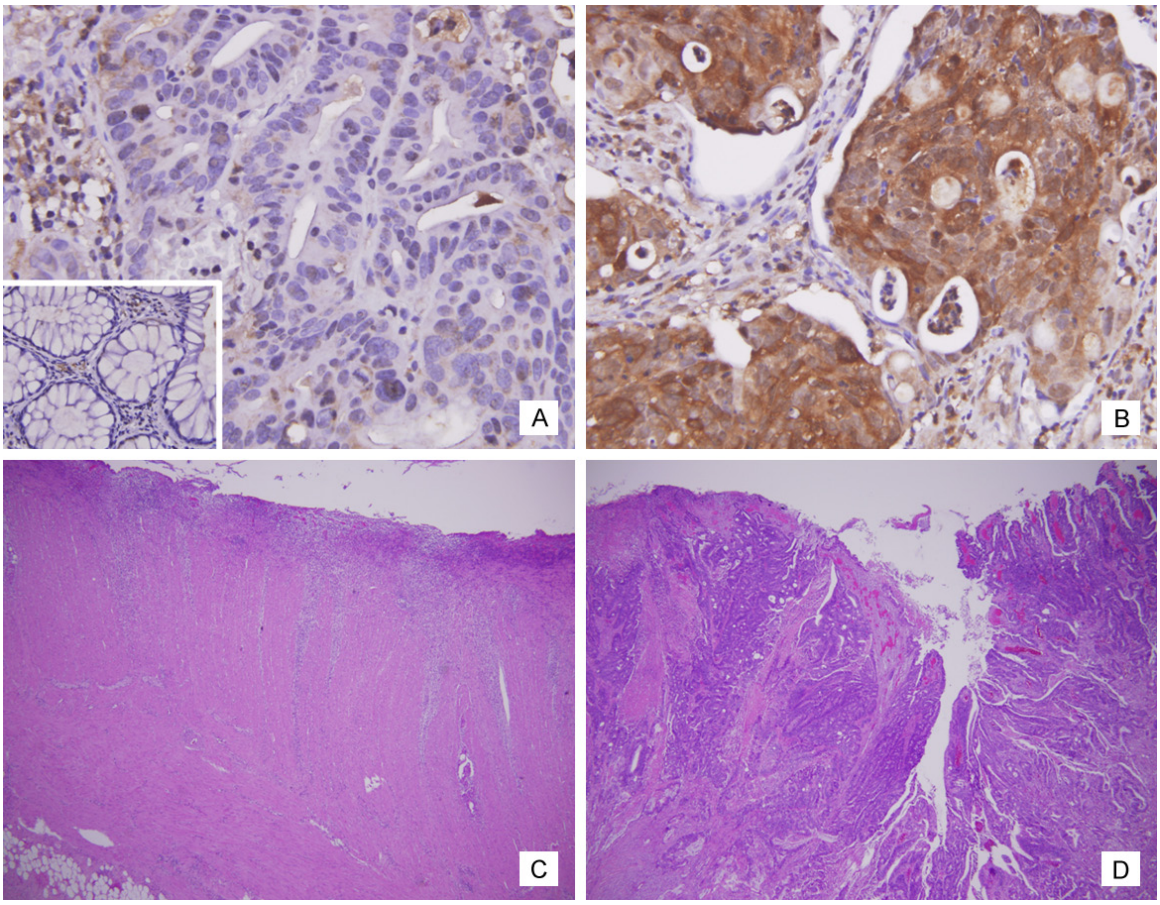

Figure 2. VNN1 immunostain on representative sections revealed (A) low VNN1 immunoreactivity was related to (B) high tumor regression grade after preoperative chemoradiotherapy; (C) high VNN1 immunoreactivity was related to (D) low tumor regression grade. Note the normal colonic mucosa with no VNN1 immunoexpression (inlet).

VNN1 expression and variable clinicopathological factors. As demonstrated in **Table 2**, VNN1 high expression was significantly associated with more advanced pre-Tx tumor invasive depth ($P < .001$), positive pre-Tx nodal metastasis ($P = .026$), more advanced post-Tx tumor invasive depth ($P < .001$) and poor response to preoperative chemoradiotherapy (**Figure 2**, $P = .001$).

Survival analyses for patients with rectal cancers

The survival analysis by univariate log-rank test was summarized in **Table 3**. More advanced post-Tx tumor status and lower tumor regression grade were significantly associated with shorter disease-specific survival (DSS), local (pelvic) recurrence-free survival (LRFS) and metastasis-free survival (MeFS) intervals (all P value ≤ .0090). Presence of vascular invasion was negatively associated with DSS and LRFS to statistical significance only ($P = .0184$ and .0028, respectively). Presence of pre-Tx nodal metastasis was significantly correlated with poorer LRFS only ($P = .0070$). Of above-mentioned prognostic indicators, only tumor regression grade was an independent prognostic factor for MeFS in multivariate analysis (**Table 4**, hazard ratio = 2.347, 95% confidence interval: 1.163-4.739, $P = .017$).

Prognostic impact of VNN1 expression on patients with rectal cancers

In univariate analysis, VNN1 overexpression was significantly associated with worse DSS ($P = .0001$), LRFS ($P = .0001$) and MeFS ($P = .0106$) (**Table 3** and **Figure 3**). In multivariate analysis, high expression of VNN1 protein still independently predicted adverse DSS (hazard ratio = 3.063, 95% confidence interval: 1.211-

Table 3. Univariate log-rank analysis for important clinicopathological variables and VNN1 expression

Parameter		No. of case	DSS		LRFS		MeFS	
			No. of event	*P*-value	No. of event	*P*-value	No. of event	*P*-value
Gender	Male	108	20	0.9026	7	0.2250	17	0.3520
	Female	64	11		20		14	
Age	< 70	106	19	0.8540	18	0.6615	20	0.7427
	≥ 70	66	12		9		11	
Pre-Tx tumor status (Pre-T)	cT1-cT2	81	10	0.0776	10	0.2261	11	0.1745
	cT3-cT4	91	21		17		20	
Pre-Tx nodal status (Pre-N)	cN0	125	19	0.0711	15	0.0070*	19	0.0973
	cN1-cN2	47	21		12		12	
Post-Tx tumor status (Post-T)	ypT0-ypT2	86	7	0.0006*	7	0.0040*	8	0.0033*
	ypT3-ypT4	86	24		20		23	
Post-Tx nodal status (Post-N)	ypN0	123	21	0.5998	16	0.1320	20	0.4634
	ypN1-ypN2	49	10		11		11	
Vascular invasion	Absent	157	25	0.0184*	21	0.0028*	27	0.4470
	Present	15	6		6		4	
Perineurial invasion	Absent	167	29	0.2559	25	0.0940	30	0.9083
	Present	5	2		2		1	
Tumor regression grade	Grade 0-1	37	13	0.0038*	10	0.0090*	14	0.0006*
	Grade 2~3	118	17		17		16	
	Grade 4	17	1		0		1	
Down stage after CCRT	Non-Sig.	150	29	0.1651	24	0.5961	30	0.0853
	Sig. (≥ 2)	22	2		3		1	
VNN1 expression	Low Exp.	86	6	0.0001*	5	0.0001*	9	0.0106*
	High Exp.	86	25		22		22	

DSS, disease-specific survival; LRFS, local recurrence-free survival; MeFS, metastasis-free survival; *, statistically significant.

Table 4. Multivariate analysis

Parameter	DSS			LRFS			MeFS		
	H.R	95% CI	*P*-value	H.R	95% CI	*P*-value	H.R	95% CI	*P*-value
Tumor regression grade	1.908	0.943-3.861	0.072	2.105	0.967-4.566	0.061	2.347	1.163-4.739	0.017*
VNN1 expression	3.063	1.211-7.745	0.018*	3.378	1.222-9.338	0.019*	1.761	0.791-3.925	0.166
Vascular invasion	2.082	0.828-5.239	0.119	2.069	0.771-5.555	0.149	-	-	-
Post-Tx tumor status (Post-T)	2.267	0.942-5.455	0.068	1.778	0.729-4.340	0.206	2.012	0.862-4.697	0.106
Pre-Tx nodal status (Pre-N)	-	-	-	1.934	0.831-4.501	0.126	-	-	-

DSS, disease-specific survival; LRFS, local recurrence-free survival; MeFS, metastasis-free survival; *, statistically significant.

7.745, $P = .018$) and LRFS (hazard ratio = 3.378, 95% confidence interval: 1.222-9.338, $P = .019$) (**Table 4**).

Discussion

Introduction of neoadjuvant concurrent chemoradiotherapy (CCRT) to patients with resectable rectal cancer not only improves survival rates, but also enhance the possibility of curative and/or sphincter-preserving surgeries due to down-staging of the tumor. The important randomized controlled studies revealed that neoadjuvant CCRT improved five-year disease-free survival rate compared with adjuvant CCRT ($P < 0.05$). Therefore, preoperative chemoradiotherapy have been gold standard treatment for patients with clinical stage II/III (cT3/4 or node-positive) rectal cancer [13]. However, colorectal cancer is still the fourth most common cause of cancer mortality, following lung cancer, liver cancer and gastric cancer [1].

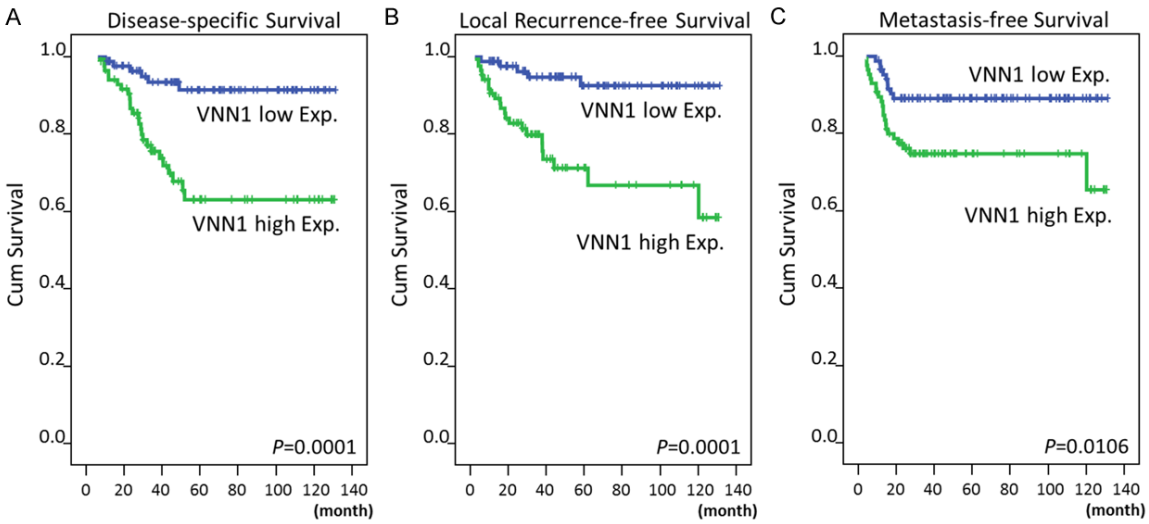

Figure 3. Kaplan-Meier survival curves showed significant prognostic impact of VNN1 expression on disease-specific survival (DSS), local recurrence-free survival (LRFS) and metastasis-free survival (MeFS) (all *P* value ≤ 0.0106).

VNN1 gene belongs to *Vanin* gene family (*VNN1-3*). All of them are located on chromosome 6q23-q24, and encode similar proteins with pantetheinase activity [7, 14]. Pantetheinase (vanin-1), the encoded protein of *VNN1* gene (A.K.A. *vanin-1* gene), was first recognized as the molecule in murine thymic stromal cell line, which was involved in pre-T cell adhesion and thymus homing [15]. Pantetheinase is a glycosylphosphatidyl inositol (GPI)-anchored protein on cell membrane and weighs 70 kDa [15]. Pantetheinase participates in synthetic pathway of pantothenic acid (vitamin B_5), by cleaving pantetheine to cysteamine and pantothenic acid. The latter is a water-soluble vitamin required for synthesis of coenzyme A (CoA). Recently, pantetheinase is also considered to play a role in the regulation of inflammation. In vanin-$1^{-/-}$ mice, glutathione-associated resistance to oxidative stress generated by whole-body gamma-irradiation or paraquat, as well as decrease of bowel inflammatory reaction induced by non-steroidal anti-inflammatory drugs and *Schistosoma mansoni* were observed [16, 17]. Production of cysteamine during the metabolic pathway of pantetheine is supposed to be the main cause of pro-inflammatory reaction. Cysteamine directly inhibits γ-glutamylcysteine synthetase, the key enzyme in the glutathione synthetic pathway [18]. Glutathione is an important antioxidant for reducing oxidative stress in organisms. On the other hand, vanin-1-deficient mice lacking cysteamine had more γ-glutamylcysteine synthetase, resulting in storage of glutathione. Hence, vanin-$1^{-/-}$ mice prevent the tissue from oxidative stress and inflammatory reaction because of increased glutathione [16, 17]. Sustained oxidative stress would induce chronic inflammation and many chronic diseases, including cancer [19, 20]. Alterations of glutathione concentration and related enzymes were also investigated in colorectal cancer and other malignancies [21-23].

Using blood sample to determine the transcriptional levels of *VNN1* and other genes as a biomarker panel, all the six genes were significantly up-regulated in patients with colorectal cancers. Among them, the fold change of VNN1 was from 1.53 to 1.87, compared with control samples ($P < .0001$) [8, 9]. Vanin-$1^{-/-}$ mice with azoxymethane injection, a carcinogenic compound for the induction of colon cancers, also showed remarkably reduced incidence of colorectal cancer [24]. Furthermore, *VNN1* and its upstream gene, *SF-1* (*NR5A1*, encoding steroidogenic factor 1), were also investigated in tumorigenesis of adrenocortical neoplasia [25, 26]. *SF-1* transgenic mice generated adrenocortical nodular hyperplasia and tumor formation rapidly. Along with the tumor progression, spreading into surrounding tissue were also observed [25]. *VNN1* transcripts were also strongly up-regulated in the adrenals of *SF-1* transgenic mice in the same study [25]. *VNN1*

has been known as the target gene of *SF-1* during the development of mouse testis [27]. Therefore, in the study of Latre de Late *et al*., *SF-1* transgenic and VNN1-null (VNN1$^{-/-}$) mice develop less neoplastic lesions in the adrenal cortex, compared to *SF-1* transgenic mice (P = .0058). Additionally, treatment of cysteamine to *SF-1* transgenic/VNN1$^{-/-}$ mice could overturn the effect (P = .0046) [26]. Consequently, the oncogenic properties of VNN1 may be due to the production of cysteamine during the synthetic pathway of pantothenic acid (vitamin B_5) and CoA. Cysteamine directly represses γ-glutamylcysteine synthetase, the rate-limiting enzyme in the synthesis of glutathione. Reduced glutathione causes uncontrolled oxidative stress and leads to an increase of reactive oxygen species in the cells, which may damage DNA and activate oncogenes [26].

Conclusively, overexpression of VNN1 (pantetheinase), an enzyme involved in vitamin B_5-CoA synthesis and regulation of inflammation and oxidative stress, was not only significantly associated with more advanced pre-Tx and post-Tx disease, but also poor response to neoadjuvant CCRT in rectal cancers. Furthermore, high VNN1 immunoreactivity in tumor cells predicted poor prognosis of patients with rectal adenocarcinoma. VNN1 overexpression was an independent negative prognosticator forecasting worse DSS and LRFS. Additional researches to elucidate the biological and molecular pathway are urgent for developing potential new therapeutic regimens targeting VNN1.

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Disclosure of conflict of interest

None.

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