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Overexpression of podocalyxin-like protein is an independent factor of poor prognosis in colorectal cancer

A Larsson[*,1], **ME Johansson**[2], **S Wangefjord**[1], **A Gaber**[1], **B Nodin**[1], **P Kucharzewska**[3], **C Welinder**[3], **M Belting**[3], **J Eberhard**[3], **A Johnsson**[3], **M Uhlén**[4,5] and **K Jirström**[1]

[1]Department of Clinical Sciences, Division of Pathology, Lund University, Skåne University Hospital, 221 85, Lund, Sweden; [2]Department of Laboratory Medicine, Center for Molecular Pathology, Lund University, Skåne University Hospital, 205 02, Malmö, Sweden; [3]Department of Clinical Sciences, Division of Oncology, Lund University, Skåne University Hospital, 221 85, Lund, Sweden; [4]Department of Proteomics, AlbaNova University Center, Royal Institute of Technology, 106 91, Stockholm, Sweden; [5]Science for Life Laboratory, Royal Institute of Technology, 106 91, Stockholm, Sweden

BACKGROUND: Podocalyxin-like 1 (PODXL) is a cell-adhesion glycoprotein and stem cell marker that has been associated with an aggressive tumour phenotype and poor prognosis in several forms of cancer. In this study, we investigated the prognostic impact of PODXL expression in colorectal cancer (CRC).

METHODS: Using tissue microarrays and immunohistochemistry, PODXL expression was evaluated in 536 incident CRC cases from a prospective, population-based cohort study. Kaplan – Meier analysis and Cox proportional hazards modelling were used to assess the impact of PODXL expression on cancer-specific survival (CSS) and overall survival (OS).

RESULTS: High PODXL expression was significantly associated with unfavourable clinicopathological characteristics, a shorter CSS (hazard ratio (HR) = 1.98; 95% confidence interval (CI) 1.38 – 2.84, $P < 0.001$) and 5-year OS (HR = 1.85; 95% CI 1.29 – 2.64, $P = 0.001$); the latter remaining significant in multivariate analysis (HR = 1.52; 95% CI 1.03 – 2.25, $P = 0.036$). In addition, in curatively resected stage III (T1 – 4, N1 – 2, M0) patients ($n = 122$) with tumours with high PODXL expression, a significant benefit from adjuvant chemotherapy was demonstrated ($p_{interaction} = 0.004$ for CSS and 0.015 for 5-year OS in multivariate analysis).

CONCLUSION: Podocalyxin-like 1 expression is an independent factor of poor prognosis in CRC. Our results also suggest that PODXL may be a useful marker to stratify patients for adjuvant chemotherapy.

Colorectal cancer (CRC) is one of the most common types of human cancer worldwide with ~ 1 million new cases detected every year (Parkin *et al*, 2005). Early detection, adequate surgical excision and optimal use of adjuvant treatment are of critical importance for the clinical outcome. Currently, tumour stage at diagnosis is the most important prognostic factor in CRC. Adjuvant treatment is recommended for patients with stage III and high-risk stage II disease (e.g. T4, tumour perforation, insufficient lymph node staging). Many efforts have been made to find molecular markers to identify high-risk disease and to select patients for adjuvant treatment, but so far, none of them have proven good enough for use in clinical routine.

Podocalyxin-like 1 (PODXL) is an anti-adhesive transmembrane protein belonging to the CD34 family. Podocalyxin-like 1 inhibits cell – cell interaction through charge-repulsive effects and has classically been ascribed to haematopoetic progenitor cells (Doyonnas *et al*, 2005), vascular endothelial cells (Horvat *et al*, 1986) and in the renal glomerular podocytes where it has a vital part in maintaining filtration pathways (Doyonnas *et al*, 2001). In recent years, overexpression of PODXL has been found in several

different forms of cancer including breast and prostate cancer, malignant brain tumours, testicular, hepatocellular and renal cell carcinoma (Gregoire *et al*, 1995; Schopperle *et al*, 2003; Chen *et al*, 2004; Somasiri *et al*, 2004; Casey *et al*, 2006; Hayatsu *et al*, 2008; Koch *et al*, 2008; Hsu *et al*, 2010; Cheung *et al*, 2011). In breast cancer and renal cell carcinoma, high expression of PODXL has been demonstrated to be an independent predictor of poor prognosis (Somasiri *et al*, 2004; Hsu *et al*, 2010). The mechanisms behind these observations are not fully understood, but PODXL has been shown to interact with other proteins such as ezrin, an established mediator of metastasis, in prostate cancer cells (Casey *et al*, 2006) and to induce actin recruitment and microvillus formation in breast cancer cells (Nielsen *et al*, 2007). In addition, a recent study demonstrated that PODXL is a target of the tumour suppressive miRNA-199a and that cellular depletion of PODXL in testicular tumours results in suppression of cancer invasion (Cheung *et al*, 2011). Podocalyxin-like 1 also has an important role in epithelial-mesenchymal transition (EMT), a process involved in initiating the invasive and metastatic behaviour of epithelial cancer cells, by regulating and interacting with collagen type 1, E-cadherin and vimentin (Meng *et al*, 2011).

Podocalyxin-like 1 has been found to be expressed in several CRC cell lines (Ito *et al*, 2007), but we are not aware of any previous publication describing the prognostic impact of PODXL expression in human CRC. In this study, we examined the

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prognostic value of PODXL expression in tissue microarrays (TMAs) representing tumours from 536 incident cases of CRC in the prospective, population-based cohort Malmö Diet and Cancer Study. The results demonstrate that PODXL expression is associated with unfavourable clinicopathological characteristics and an independent predictor of poor prognosis.

MATERIALS AND METHODS

The Malmö Diet and Cancer Study

The Malmö Diet and Cancer Study (MDCS) is an ongoing population-based prospective cohort study with the primary aim to examine whether a Western diet rich in fat and low in fruit and vegetables increases the risk of certain forms of cancer (Berglund *et al*, 1993). Between 1991 and 1996, a total number of 28 098 individuals, 11 063 (39.4%) men and 17 035 (60.6%) women between 44 and 74 years where enrolled (from a background population of 74 138). Follow-up is done annually by record linkage to national registries for cancer and cause of death.

Approval for the MDCS (Ref. 51/90) and the present study (Ref. 530/2008) were obtained from the Ethics Committee at Lund University.

Incident cases of CRC until 31 December 2008

Until the end of follow-up 31 December 2008, 626 incident cases of CRC had been registered in the study population. Cases were identified from the Swedish Cancer Registry up until 31 December 2007, and from The Southern Swedish Regional Tumour Registry for the period of 1 January to 31 December 2008. All tumours with available slides and/or paraffin blocks were histopathologically re-evaluated on haemotoxylin and eosin-stained slides. Histopathological, clinical and treatment data were obtained from clinical and/or pathology records. TNM staging was performed according to the American Joint Committee on Cancer (AJCC).

Information on vital status and cause of death were obtained from the Swedish Cause of Death Registry up until 31 December 2009. Follow-up started at the date of diagnosis and ended at death, emigration or 31 December 2009, whichever came first. Patient and tumour characteristics are summarised in Supplementary Table 1.

TMA construction

Tumours with an insufficient amount of material were excluded, and a total number of 557 (89%) tumours were suitable for TMA construction. Areas representative of cancer were then marked on haematoxylin and eosin-stained slides and TMAs were constructed as previously described (Kononen *et al*, 1998). In brief, two 1.0 mm cores were taken from each tumour and mounted in a new recipient block using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminster, MD, USA).

Immunohistochemistry and antibody validation

For immunohistochemical analysis, 4 μm TMA sections were automatically pre-treated using the PT-link system (DAKO, Glostrup, Denmark) and then stained in an Autostainer Plus (DAKO) with the affinity-purified polyclonal anti-PODXL antibody HPA 2110 (Atlas Antibodies, Stockholm, Sweden, diluted 1:250). The Envision Flex/HRP (K8010) kit (DAKO) was used for visualisation of the staining. The specificity of this antibody, originally generated within the Human Protein Atlas (HPA) project, has been validated using western blotting and protein arrays and PODXL protein expression has been mapped by immunohistochemistry in 48 types of normal tissues and 20 common cancers (Uhlen *et al*, 2005; Ponten *et al*, 2008) (www.proteinatlas.org). The same antibody has also been used to detect PODXL expression in testicular carcinoma in a recent study (Cheung *et al*, 2011).

To control for heterogenous expression patterns, IHC was also performed on full-face sections from 10 randomly selected cases denoted as having negative PODXL expression and 10 cases with high (score 3–4) PODXL expression.

Evaluation of PODXL staining

Podocalyxin-like 1 was expressed in the cytoplasm of the tumour cells, with an accentuation towards the membrane in some cases. No expression was seen in the nuclei. The expression was recorded as negative (0), weakly positive in any proportion of cells (1), moderately positive in any proportion (2), positive with distinct membranous pattern in $\leqslant 50\%$ of cells (3) and positive with distinct membranous pattern in $> 50\%$ of cells (4). The staining was evaluated by two independent observers (AL and KJ) who were blinded to clinical and outcome data. Scoring differences were discussed in order to reach consensus.

Statistical analysis

For statistical purposes, categories of PODXL expression were trichotomised into negative (0), weak-moderate (1–2) and strong (3–4) PODXL staining or dichotomised into low (0–2) and high (3–4). Spearman's Rho and χ^2-tests were used for comparison of PODXL expression and relevant clinicopathological characteristics. Kaplan–Meier analysis and log rank test were used to illustrate differences in cancer specific survival (CSS) and overall survival (OS) according to PODXL expression. Cox regression proportional hazards models were used for estimation of hazard ratios (HRs) for death from CRC and overall causes according to PODXL expression in both uni- and multivariate analysis, adjusted for age, gender, TNM status, differentiation grade and vascular invasion. A backward conditional selection method was used for variable selection by the model. The interaction between PODXL expression and adjuvant chemotherapy was explored by a Cox model including a treatment variable and an interaction variable. All tests were two-sided. A P-value < 0.05 was considered significant. All statistical analyses were performed using SPSS version 18 (SPSS Inc., Chicago, IL, USA).

REMARK criteria

A description of the fulfilment of REMARK criteria (McShane *et al*, 2005) for biomarker studies is provided in Supplementary Table 2.

RESULTS

PODXL expression in CRC

Following antibody optimisation and staining, PODXL expression could be evaluated in 536 of the 557 (96.2%) of the tumours represented in the TMA. A total of 268 tumours (50.0%) were negative for PODXL, 196 (36.6%) displayed weak-moderate staining and 72 (13.4%) strong staining for PODXL. As endothelial cells express PODXL, entrapped vessels served as internal positive control. Representative IHC images are shown in Figure 1 A–F. Notably, in the majority of cases denoted as positive, PODXL was distinctly expressed in scattered invasive cells at the tumour front, corresponding morphologically with tumour budding (Prall, 2007) (Figure 1D and E). Only one tumour (0.2%) had a strong, distinct positive staining in >50% of tumour cells, as visualised in Figure 1F. Podocalyxin-like 1 expression in full-face sections ($n = 20$) correlated with the TMA-based scoring in all cases, including the tumour in Figure 1F, further supporting the selective distribution of PODXL-expressing cells in the majority of positive tumours.
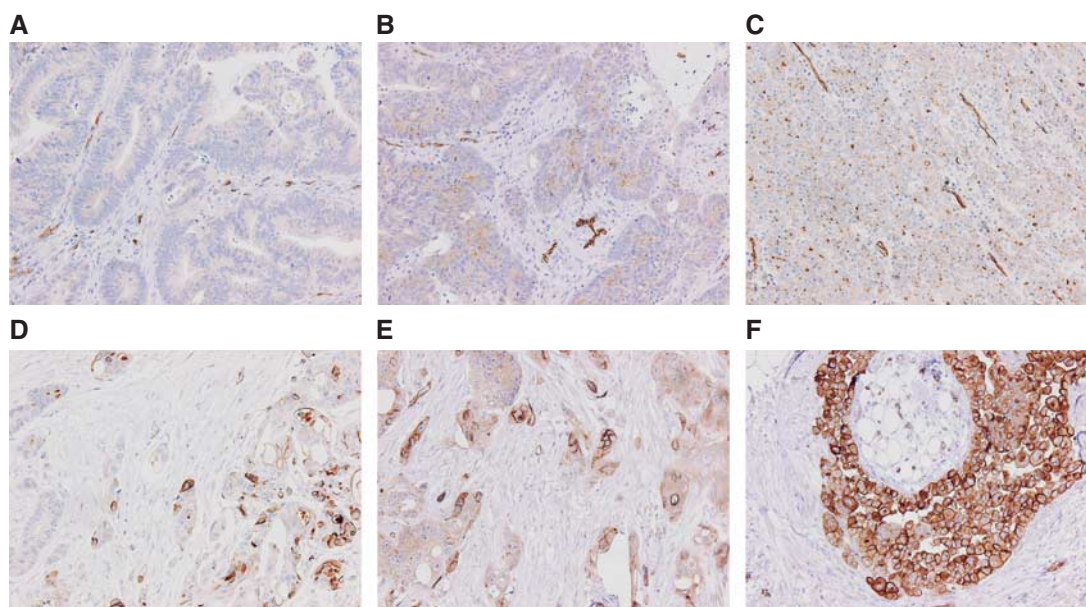
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Figure I Immunohistochemical images of PODXL staining representing colorectal tumours with (**A**) negative, (**B**) weak, (**C**) moderate, and (**D–F**) strong, membranous staining in a varying proportion of tumour cells. All images are captured at $\times 20$ magnification.

Association between PODXL expression and clinicopathological characteristics

Analysis of the relationship between PODXL expression and established clinicopathological parameters revealed a strong correlation between high PODXL expression and more advanced T-stage ($P < 0.001$), N-stage ($P < 0.001$), M-stage ($P = 0.009$), low differentiation grade ($P < 0.001$) and presence of vascular invasion ($P = 0.008$) (Table 1). There was no significant association between PODXL expression and age at diagnosis, gender or tumour location (Table 1).

Association between PODXL expression and survival

Kaplan–Meier analysis revealed that PODXL expression correlated with a significantly shorter CSS and OS, with the worst outcome for tumours with high PODXL expression (Figure 2). These findings were confirmed in univariate Cox regression analysis using a dichotomised variable of low (0–2) and high (3–4) PODXL expression (HR = 1.98; 95% confidence interval (CI) 1.38–2.84, $P < 0.001$ for CSS and HR = 1.85; 95% CI 1.29–2.64, $P = 0.001$ for 5-year OS), and remained significant for 5-year OS in multivariate analysis (HR = 1.52; 95% CI 1.03–2.25, $P < 0.036$) and borderline significant for CSS (HR = 1.57; 95% CI 0.99–2.18, $P = 0.055$), adjusted for age, gender, TNM status, differentiation grade and vascular invasion (Table 2). Podocalyxin-like 1 expression was also significantly associated with long-term OS, both in univariate analysis (HR = 1.97; 95% CI 1.41–2.74, $P < 0.001$) and multivariate analysis (HR = 1.57; 95% CI 1.10–2.25, $P = 0.014$).

Impact of PODXL expression on survival in relation to adjuvant chemotherapy

The impact of PODXL expression on survival in relation to adjuvant treatment was analysed in 122 curatively resected stage III (T1–4, N1–2, M0) patients, of whom 62 (50.8%) had received adjuvant treatment and 60 (49.2%) had not. Kaplan–Meier analysis in strata according to treatment and PODXL expression demonstrated that patients having tumours with high PODXL expression who were treated with adjuvant chemotherapy had a similar CSS (Figure 3A) and OS (Figure 3B) as patients with low

PODXL-expressing tumours. Untreated patients with tumours with high PODXL expression, however, had a significantly shorter CSS and OS (Figure 3A and B). Cox interaction analysis (Table 3) demonstrated that the *P*-value for the unadjusted interaction variable between treatment and PODXL status was 0.044 for 5-year OS, suggesting that patients having tumours with high expression of PODXL had benefited from adjuvant chemotherapy, whereas patients with low PODXL-expressing tumours did not benefit from adjuvant treatment. In addition, when adjusted for established prognostic factors, the term of interaction was significant for both CSS ($P = 0.004$) and 5-year OS ($P = 0.015$) (Table 3). The treatment benefit was similar for 5-fluorouracil alone or in combination with oxaliplatin (data not shown).

In patients with stage II disease ($n = 205$), high PODXL expression was associated with a significantly shorter OS (HR = 3.03; 95% CI 1.45–6.34, $P = 0.003$) and 5-year OS (HR = 2.83; 95% CI 1.14–7.16, $P = 0.025$), whereas the association with CSS did not reach significance (HR = 2.19; 95% CI 0.84–5.75, $P = 0.11$). Information on adjuvant chemotherapy was available for 170 patients with stage II disease, of whom only 13 (6.2%) had received treatment, an insufficient number to allow for analysis of a potential treatment benefit related to PODXL status in this group.

DISCUSSION

Our results demonstrate that overexpression of PODXL in CRC is associated with unfavourable clinicopathological characteristics and an independent factor of poor prognosis. This is to our knowledge the first report on the prognostic value of PODXL expression in human CRC and the results are based on a large number of patients ($n = 536$) from a prospective cohort study.

In our study, 13% of the tumours were denoted as having high expression of PODXL. In a previous study on breast cancer, using a somewhat different cutoff, high expression of PODXL was found in 6% of the tumours (Somasiri *et al*, 2004). These data, together with the results from the antibody-based screening in the Human Protein Atlas, demonstrate that, in most cancer types, while upregulated compared with the corresponding normal tissue, PODXL is overexpressed in a relatively small proportion of cases.

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Table 1 Association between PODXL expression and clinicopathological parameters

n (%)	PODXL expression			
	Negative 268 (50.0)	Weak-moderate 196 (36.6)	Strong 72 (13.4)	P-value
Age				
≤75	186 (69.4)	137 (69.9)	45 (62.5)	0.484
>75	82 (30.6)	59 (30.1)	27 (37.5)	
Gender				
Female	131 (48.9)	110 (56.1)	38 (52.8)	0.209
Male	137 (51.1)	86 (43.9)	34 (47.2)	
T-stage[a]				
1	32 (12.5)	13 (6.8)	1 (1.4)	<0.001**
2	36 (14.1)	21 (11.0)	7 (10.0)	
3	157 (61.6)	130 (68.1)	40 (57.1)	
4	30 (11.8)	27 (14.1)	22 (31.4)	
Missing	13	5	2	
N-stage[a]				
0	157 (64.6)	103 (56.9)	26 (37.7)	<0.001**
1	53 (21.8)	48 (26.5)	21 (30.4)	
2	33 (13.6)	30 (16.6)	22 (31.9)	
Missing	25	15	3	
M-stage[a]				
0	228 (86.4)	157 (80.9)	52 (73.2)	0.009**
1	36 (13.6)	37 (19.1)	19 (26.8)	
Missing	4	2	1	
Differentiation grade				
High	21 (8.0)	12 (6.3)	1 (1.4)	<0.001**
Intermediate	202 (76.8)	140 (72.9)	31 (43.7)	
Low	40 (15.2)	40 (20.8)	39 (54.9)	
Missing	5	4	1	
Vascular invasion				
No	85 (55.2)	52 (45.6)	15 (33.3)	0.008**
Yes	69 (44.8)	62 (54.4)	30 (66.7)	
Missing	114	82	27	
Location				
Colon	160 (59.9)	117 (60.0)	49 (68.1)	0.300
Rectum	98 (36.7)	74 (37.9)	23 (31.9)	
Multiple	9 (3.4)	4 (2.1)	0 (0)	
Missing		1	0	

**Significant at the 0.01 level. [a]TNM staging was performed according to the AJCC classification system: T1 = tumor invades submucosa; T2 = tumor invades muscularis propria; T3 = tumor invades through the muscularis propria into the subserosa or into non-peritonealized pericolonic or perirectal tissues; T4 = tumor penetrates the visceral peritoneum or directly invades or is adherent to other organs or structures; No = no regional lymph node metastasis; N1 = metastasis in 1 to 3 regional lymph nodes; N2 = metastasis in four or more regional lymph nodes; M0 = no distant metastasis; M1 = distant metastasis.

In the vast majority of tumours with high PODXL expression, defined as a distinct cytoplasmic/membranous staining pattern, this was predominantly observed in a subset of scattered and infiltrating cells at the invasive tumour front. These findings are substantiated by previous studies demonstrating that overexpression of PODXL enhances migratory and invasive properties of tumour cells (Casey et al, 2006; Nielsen et al, 2007). Notably, the only patient in this study with a tumour displaying PODXL expression in >50% of the cells had generalised disease and died within a month of diagnosis, further supporting that PODXL expression is associated with an invasive and metastatic cellular phenotype in CRC *in vivo*. Moreover, the upregulation of PODXL expression observed at the infiltrating edge of the tumour
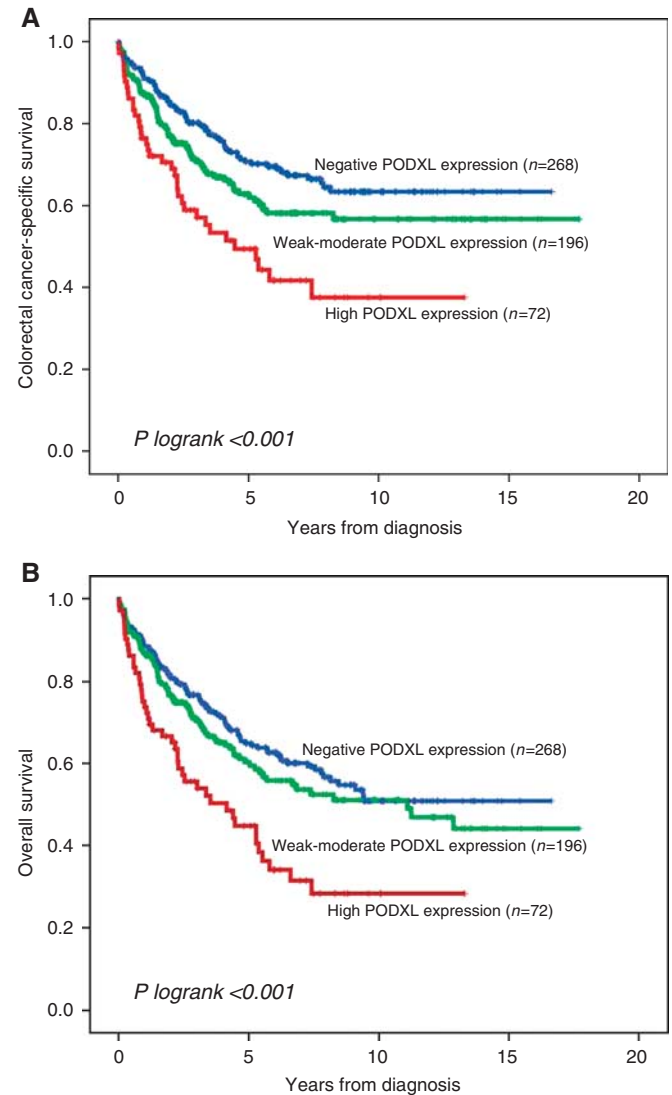

Figure 2 High expression of PODXL is associated with a poor outcome in colorectal cancer patients. Kaplan–Meier analysis of (**A**) colorectal cancer-specific survival and (**B**) overall survival according to PODXL expression.

corresponds well to the morphological term 'tumour budding', which has been demonstrated to be of prognostic importance (Hase et al, 1993; Prall, 2007), and biologically closely related to EMT (Prall, 2007). Studies have demonstrated that cancer cells at the invasive front of primary tumours can convert to a motile mesenchymal phenotype (Yang and Weinberg, 2008) and these cells are considered to be the cells with metastatic potential. Along this line, a high expression of PODXL has been recorded in TGFβ-induced EMT (Meng et al, 2011) indicating that PODXL has an integral role in cancer progression.

Moreover, studies have illustrated a link between EMT and the gain of stem cell-like properties such as mobility, anti-apoptosis circuitry and low level of differentiation, which are traits of great value for a malignant tumour (Mani et al, 2008). The PODXL has previously been suggested as a marker for embryonal carcinoma stem cells and haematopoietic stem cells (Schopperle et al, 2003) and found to be upregulated in tumour stem cells in glioblastoma (He et al, 2010).

In the light of these results, the development of therapies targeting PODXL has been proposed as a new treatment strategy (Chen et al, 2004; Somasiri et al, 2004). Our results, however,

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Table 2 Cox uni- and multivariate analysis of relative risks of death from colorectal cancer and overall causes within 5 years according to PODXL expression

	Colorectal cancer-specific survival			5-year overall survival		
	HR (95% CI)	P-value	n (events)	HR (95% CI)	P-value	n (events)
	Univariate					
PODXL low	1.00		464 (147)	1.00		464 (153)
PODXL high	1.98 (1.38−2.84)	<0.001	72 (37)	1.85 (1.29−2.64)	0.001	72 (37)
	Multivariate					
PODXL low	1.00		417 (126)	1.00		417 (126)
PODXL high	1.57 (0.99−2.18)	0.06	68 (34)	1.52 (1.03−2.25)	0.036	68 (34)

Abbreviations: CI = confidence interval; HR = hazard ratio. Multivariate analysis included adjustment for age (>/⩽75 years), gender, T-stage (I−II, III, IV), N-stage (0, 1, 2), M-stage (0, 1) differentiation grade (high-intermediate *vs* low) and vascular invasion (absent, present, missing).
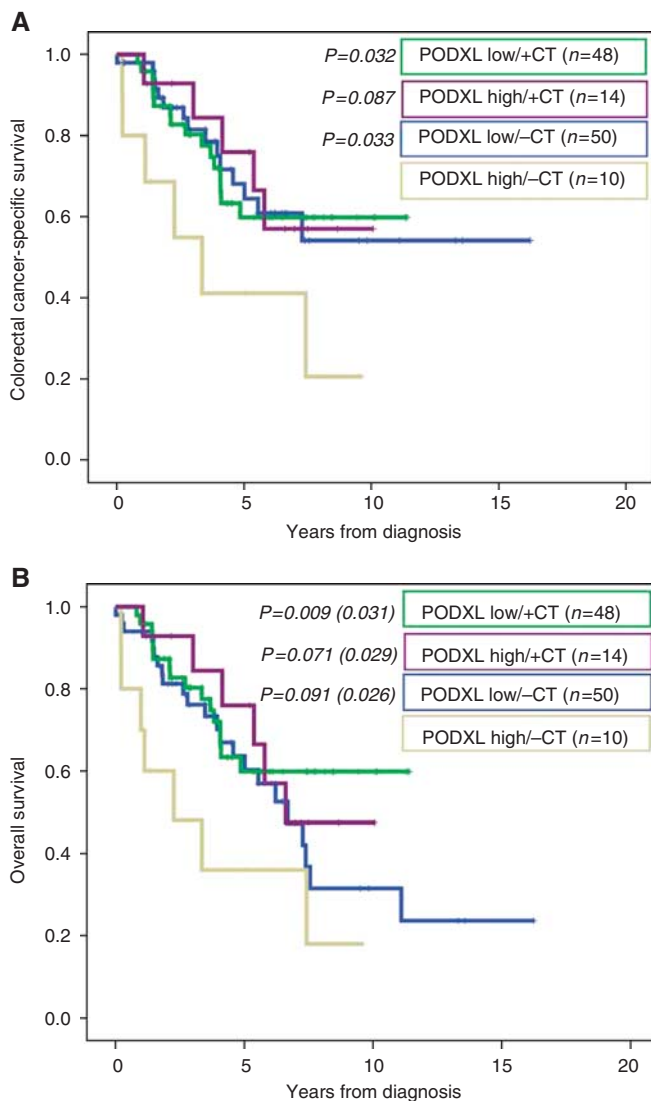
Figure 3 Kaplan−Meier estimates of (**A**) colorectal cancer-specific and (**B**) overall survival according to combinations of PODXL expression (high or low) and adjuvant chemotherapy (CT). Log rank *P*-values correspond to pairwise comparisons of PODXL high and untreated tumours with the other strata. Log rank *P*-values for overall survival (**B**) are given both for 5-year survival (in parentheses) and long-term survival.

indicate that colorectal tumours with high expression of PODXL also respond well to standard chemotherapy. Adjuvant chemotherapy reduces the risk of relapse but it also has side effects and can be difficult to tolerate, especially for older patients. In CRC Stage III, the recurrence-free survival increases with 10–20% by adding adjuvant chemotherapy and in stage II the incremental gain is only around 5%. Thus, only a minority of patients receiving adjuvant chemotherapy have any benefit from it. Therefore, it would be of great clinical value to find predictive tools for a better selection of patients to such treatment.

The present study indicates that PODXL might be used as a predictive marker for adjuvant treatment of CRC. In stage III, patients with high tumour PODXL expression were the ones who benefited from adjuvant chemotherapy, whereas patients having tumours with low PODXL expression did not seem to have any effect from the treatment. Whether the same is true in stage II could not be determined in our study, as the number of stage II patients that received adjuvant chemotherapy was too low for statistical analyses.

Even though the positive impact of chemotherapy in the tumours with high PODXL expression remained significant in multivariate analysis after correction for other prognostic factors, our findings must be interpreted with caution as this was an observational cohort study in which a potential selection bias compared with the general population must be taken into consideration (Berglund *et al*, 1993). The distribution of clinical stages at diagnosis was, however, in line with the expected.

It is notable that only ∼half of the patients with stage III tumours in our study received adjuvant treatment, which is on the low side compared with modern therapeutic guidelines. The main reason for this is most likely that this study started already in the mid 90s, during which time adjuvant chemotherapy was not yet standard of care in Sweden.

In this study, CRC-specific survival and OS were used as endpoints as this information had been recorded prospectively and therefore was available for all patients. In future studies, the impact of PODXL expression on recurrence free survival should also be assessed, preferably in cohorts where this information has been recorded prospectively. It will also be of value to correlate PODXL expression to other relevant molecular characteristics of CRC, for example, microsatellite instability status, KRAS, BRAF and PIK3C mutations.

In summary, the results from this large cohort study suggest that PODXL expression is independently associated with a poor prognosis in CRC. Our findings also suggest that adjuvant chemotherapy should be given to patients with tumours expressing high levels of PODXL, whereas patients with low PODXL-expressing tumours could be spared such treatment. Future prospective studies are needed to confirm this association as well

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Table 3 Cox proportional hazards model for colorectal cancer-specific survival and 5-year overall survival according to adjuvant chemotherapy and PODXL expression in curatively treated patients with Stage III (T1−4/N1−2/M0) disease

	Colorectal cancer-specific survival					5-year overall survival				
	HR (95% CI)	n (events)	P*	P**	P†	HR (95% CI)	n	P*	P**	P†
PODXL low										
Untreated	1.00	50 (15)				1.00	50 (15)			
Treated	1.03 (0.51−2.09)	48 (16)	0.924			1.04 (0.51−2.10)	48 (16)	0.920		
PODXL high										
Untreated	1.00	10 (6)				1.00	10 (6)			
Treated	0.36 (0.11−1.21)	14 (5)	0.100	0.107	0.004	0.24 (0.06−0.96)	14 (3)	0.044	0.041	0.015

Abbreviations: CI = confidence interval; HR = hazard ratio. *P-value from univariate Cox regression analysis. **P-value for term of interaction by Cox multivariate analysis including adjuvant chemotherapy, the binary covariate PODXL expression and a term of interaction. †P-value for term of interaction adjusted for T-stage (1−2 vs 3 and 4), N-stage (1 vs 2), age (>/⩽ 75 years), differentiation grade (high-intermediate vs low) and vascular invasion (absent, present, missing).

as to elucidate the exact mechanisms by which PODXL affects tumour behaviour.

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