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Research Article

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Circulating Levels of the Wnt Antagonist Dkk-3 as a Diagnostic Marker for Colorectal Cancer

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Abstract

The Wnt antagonist Dickkopf-3 (Dkk-3) has been implicated in several stages of tumour development in a wide range of human cancers, including colorectal cancer (CRC). However, the usefulness of serum Dkk-3 levels as a diagnostic biomarker for CRC has yet to be determined. In this study we used an ELISA immunoassay to examine serum Dkk-3 protein levels in a retrospective cohort of CRC patients (n = 89) and age, gender matched controls (n = 46). The median concentration of Dkk-3 was significantly (p = 0.0003) lower in CRC patient serum samples (29.3 ng/ml, range 10.4 – 67.8 ng/ml) when compared to control serum samples (36.8 ng/ml, range 20.7 – 67.4 ng/ml). Receiver operating characteristic analysis demonstrated at 90% specificity, serum Dkk-3 levels distinguished CRC patients with 36% sensitivity (AUC = 0.69, 95% CI 0.60 – 0.78).

Keywords: Colorectal cancer; Diagnostic biomarker; Dickkopf-3; Dkk-3

Abbreviations: Dkk-3: Dickkopf-3; CRC: Colorectal Cancer; APC: Adenomatous Polyposis Coli; TcF: T cell Factors; Lrp: Lipoprotein Receptor-related Protein; Krm: Kremen; βTrCP: β-transducin Repeat-containing Protein; ELISA: Enzyme-linked Immunosorbent Assay; BSA: Bovine Serum Albumin; AUC: Area under the Curve; ROC: Receiver Operating Curve

Introduction

Colorectal cancer remains as one of the most frequently diagnosed cancers worldwide and while its prevalence is highest amongst affluent countries (e.g., US, UK, Australia, Europe), it appears to be increasing in traditionally low risk countries that are becoming more affluent (e, Asian countries such as Japan and Korea) [1]. Studies of both inherited and sporadic colorectal cancer (CRC) have demonstrated that dysregulated activation of the Wnt pathway is central to colorectal carcinogenesis [2]. One of the earliest events in colorectal tumorigenesis is loss of Adenomatous polyposis coli (APC) [3] gene function which leads to the accumulation of stabilised cytoplasmic β-catenin that can then enter the nucleus and act as a co-activator of T cell factors (Tcf) enabling transcription of Wnt target genes [4,5]. The Wnt pathway is regulated by both intracellular and extracellular modulators, and due to its role in carcinogenesis, potential antagonists of this pathway have received much attention as candidate anti-cancer drugs [6].

The Dickkopf (Dkk) family of secreted proteins is one such group of extracellular Wnt antagonists, and consists of four main members Dkk1-4 and the Dkk-3-related protein Dkkl1 [7]. Dkk-1, Dkk-2, and Dkk-4 bind and inhibit the Wnt co-receptors, low-density lipoprotein receptor-related protein (Lrp) 5 and 6, with high affinity to antagonise Wnt/β-catenin signalling [7]. They can further modulate Wnt signalling by binding Kremen (Krm) 1 and 2 to form a complex that controls the internalization and degradation of Lrp [7]. Dkk-3 is the least characterised member of the Dkk family, however its emerging role in carcinogenesis has lead to increased interest into how this protein functions to inhibit the Wnt pathway and has recently been reviewed [8]. Dkk-3 differs from the other Dkk family proteins as it does not interact with Lrp5/6, and does not bind with the Krm on the cell surface [9] but rather intracellularly [10]. Despite this, Dkk-3 appears to prevent the nuclear accumulation of β-catenin [11] and decrease Tcf-driven gene expression of Wnt target genes [12]. Furthermore, Dkk-3 can co-localise with β-transducin repeat-containing protein (βTrCP) to directly target β-catenin degradation [13]. However, the precise mechanisms underlying these processes are yet to be determined. The tumour suppressor role of Dkk-3, in its ability to inhibit cancer cell growth, is more clearly understood and has lead to interest in its potential use as a therapeutic target. In vitro studies of isolated cell lines of multiple lineages have shown that Dkk-3 can induce apoptosis via caspase-3 cleavage and the ER stress pathway [14,15]. *In vitro* and clinical studies have shown that Dkk-3 expression has been shown to be downregulated by hypermethylation of the DKK3 gene promoter [12,16-19] in a wide range of human cancer types [16,19-23], including those of the gastrointestinal tract [16]. Furthermore, methylation of DKK3 is associated with adverse patient outcome in acute lymphoblastic leukemia [20], breast cancer [22], liver cancer [23] and gastric cancer [16], but surprisingly not CRC [16]. Interestingly, in CRC a role for Dkk-3 in neoangeogenesis has been described [24,25].

While several reports have examined tissue or circulating levels of methylated Dkk-3 in cancers, including CRC [26,27] none have measured Dkk-3 protein levels in the blood of CRC patients. Here we

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describe a retrospective, age and gender matched, case-controlled study utilizing an ELISA immunoassay to examine serum Dkk-3 protein levels and assess its potential usefulness as a blood-based biomarker for the diagnosis of CRC.

Methods

Serum samples

Serum samples were collected from healthy donors (n = 46) and CRC patients (n = 89) following initial diagnosis of the disease using serum separator tubes. Patients with a previous history of CRC or who had received chemo- and/or radio-therapy were excluded from the study. Blood samples were collected at preadmission clinics from a network of hospitals in Melbourne, Victoria, Australia, between 2005 and 2009.

All serum samples were labeled with a unique identifier to protect confidentiality and processed at a centralized location, following a standardized protocol within two hours of collection. Blood was incubated at room temperature for at least 30 minutes to allow clot formation. Samples were then centrifuged at 1200 g for 10 minutes at room temperature and serum transferred to a 15 ml polypropylene tube. To remove any possible suspended cells or cell debris, serum samples underwent an additional centrifugation at 1800 g for 10 minutes at room temperature prior to storage at -80°C as 250 µl aliquots until analysis. Serum samples were subjected to no more than one freeze thaw cycle. This study was approved by human research ethics committees at CSIRO Adelaide and the Victoria Cancer Biobank, Melbourne.

Enzyme-linked immunosorbent assay

Serum levels of Dkk-3 were measured by human Dkk-3 DuoSet enzyme-linked immunosorbent assay (ELISA) according to the following protocol (R&D Systems, Minneapolis, USA). Briefly, 96 well Maxisorb microtitre plates (Nunc, Roskilde, Denmark) were coated with 2 µg/ml mouse anti-human Dkk-3 capture antibody overnight prior to being washed thrice with 0.05% Tween-20/1xPBS. The plates were then blocked with 1% (w/v) bovine serum albumin (BSA)/1xPBS for 2 hours. Wells were washed another three times with 0.05% Tween-20/1xPBS and incubated with serum samples diluted 1:40 in 1%BSA/1xPBS for 2 hrs at room temperature with gentle shaking. Wells were washed three times with 0.05% Tween-20/1xPBS and biotinylated goat anti-human Dkk-3 detection antibody was added at 200 ng/ml for 2 hrs at room temperature with gentle shaking. Unbound detection antibody was removed by washing three times with 0.05% Tween-20/1xPBS and strepavidin-conjugated to horseradish peroxidase (R&D Systems, Minneapolis, USA) added for 20 minutes at room temperature with gentle shaking. Wells were again washed three times with 0.05% Tween-20/1xPBS prior to colour development with substrate reagent, equal parts hydrogen peroxide and tetramethylbenzine (R&D Systems, Minneapolis, USA). The colorimetric reaction was stopped by the addition of 2N H_2SO_4 and absorbance read at 450 nm with wavelength correction at 600 nm on a Wallac Victor3V Multilabel Counter plate reader (Perkin Elmer, Massachusetts, USA).

Two in-house quality control samples were included as part of this analysis. These samples consisted of a pooled control sample (n = 41) and a pooled CRC sample (n = 41). Standards and samples were analyzed in duplicate. The standard curve ranged from 1.25 - 80 ng/mL, and the intra-assay coefficient of variation was <10%. Standard curves were generated using 5-parameter curve fit and serum Dkk-3 concentrations calculated using Workout 2.0 software (Dazdaq, East Sussex, UK) (Table 2).

Statistical analysis

Data were analysed using GraphPad Prism 5, version 5.02 (GraphPad, California, USA). Mann-Whitney tests were used to determine statistical significances between healthy control and CRC patient Dkk-3 levels. Kruskal-wallis with Dunn's multiple comparison tests was used to determine statistical significances between controls and CRC patient Dkk-3 levels stratified by Dukes' staging. Receiver Operator Curve (ROC) curves was generated to quantify the ability of serum Dkk-3 levels to discriminate between healthy controls and those with CRC.

Results

Patient characteristics

The characteristics of the patient cohort used in this study are summarised in Table 1. The median donor age of CRC patients was 68 years (range 44 - 93yrs), and included 21 patients with Dukes' stage A tumours (median age 66 yrs, range 44 – 93 yrs), 28 patients with Duke's stage B tumours (median age 68 yrs, range 47 – 93 yrs), 32 patients with Duke's stage C tumours (median age 69 yrs, range 46 – 81 yrs), and 8 patients with Duke's stage D tumours (median age 71 yrs, range 46 – 85 yrs). The median donor age of the healthy cohort was 70 yrs (range 50 – 85 yrs).

Serum levels of Dkk-3 are decreased in CRC patients

Dkk-3 protein was detected in serologic samples from both healthy control and CRC patients. The median (range) serum levels of Dkk-3 was 36.8 ng/ml (20.7– 67.4 ng/ml) in the healthy controls and 29.3 ng/ml (10.4 – 67.8 ng/ml) in the CRC patients. Serum levels of Dkk-3 were significantly lower in CRC patients than in healthy controls (p=0.0003) (Figure 1A). Median serum Dkk-3 levels in patients with cancers of Dukes stages A, B and C were all significantly lower than for healthy controls while a similar trend was observed in patients with stage D

			Dukes' stage			
Characteristics	**Control**	**CRC**	**A**	**B**	**C**	**D**
N	46	89	21	28	32	8
Median age, yrs (range)	70 (50-85)	68 (44-93)	66 (44-93)	68 (47-93)	69 (46-81)	71 (46-85)
Gender, N						
Female	23	46	10	14	17	5
Male	23	43	11	14	15	3
Gender, median age, yrs (range)						
Female	70 (51-85)	67 (46-93)	67 (58-85)	68 (47-93)	71 (51-81)	62 (46-84)
Male	70 (50-84)	68 (44-93)	66 (44-93)	68 (52-85)	68 (46-79)	79 (62-85)

Table 1: Age, gender and clinical characteristics of the cohort used for the enzyme-linked immunosorbent assay detection of Dkk-3 protein levels in serum samples.

Diagnostic subgroup	Area under the ROC curve (95% CI)	Sensitivity at 90% Sensitivity (95% CI)
CRC	0.69 (0.60 - 0.78)	36% (26-47%)
Dukes' A	0.59 (0.44 - 0.75)	24% (8-47%)
Dukes' B	0.68 (0.55 - 0.82)	32% (16-52%)
Dukes' C	0.74 (0.62 - 0.86)	47% (29-65%)
Dukes' D	0.77 (0.62 - 0.93)	38% (9-75%)

Abbreviations: CI, confidence interval; ROC, receiver-operating characteristic; CRC, colorectal cancer encompassing all Duke's stages

Table 2: Receiver operator characteristic analysis of serum Dkk-3 levels between CRC patients and healthy controls.

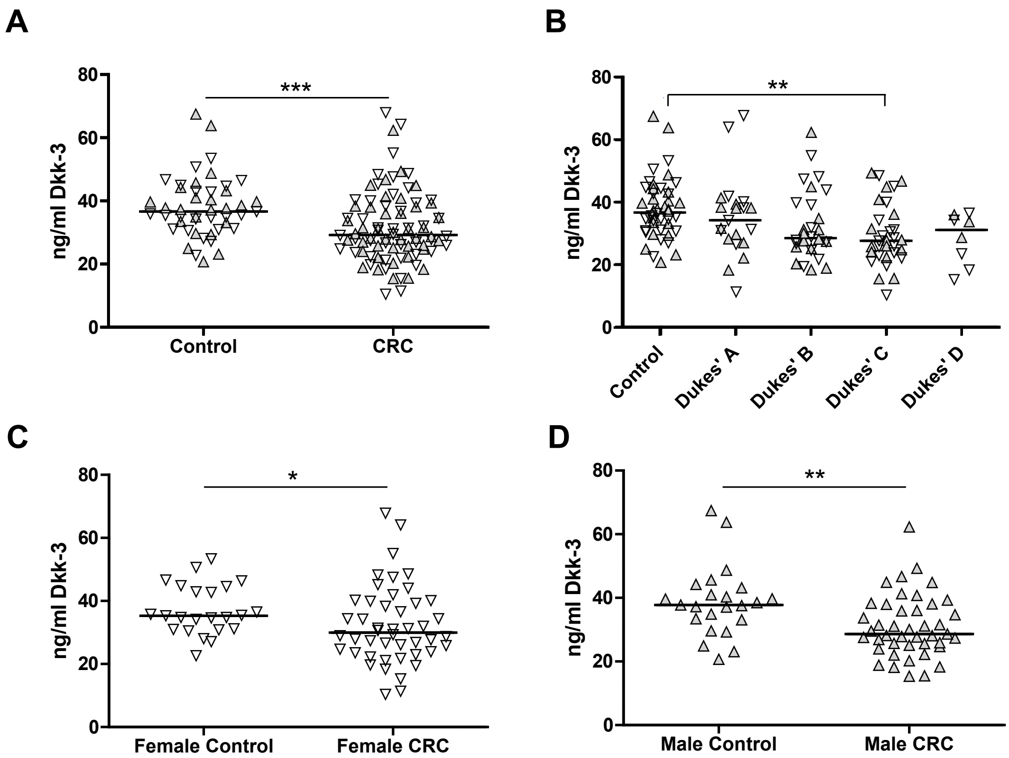

Figure 1: A. Serum Dkk-3 protein levels measured by ELISA were decreased in CRC patients compared to healthy controls (*p*=0.0003). B Serum Dkk-3 protein levels did not significantly differ between CRC disease stages. However, CRC patients with stage C tumours had significantly decreased serum Dkk-3 levels compared to healthy controls (*p*<0.01). C. Female CRC patients had significantly lower serum Dkk-3 levels than healthy females (*p*=0.024). D. Likewise, male CRC patients had significantly lower serum levels than healthy males (*p*=0.005). Individual results are indicated by scatter plots and horizontal bars represent the median values. Females are denoted by [▽], males are denoted by [▲].

cancers, but this did not reach significance (Figure 1B). This reduced level of serum Dkk-3 was particularly marked in patients with Stage C tumours (p<0.01) (Figure 1B). There was no significant difference between serum levels of Dkk-3 between cancer stages. Both, female (p = 0.024) and male (p = 0.005) CRC patients had lower serum Dkk-3 levels than their corresponding healthy counterparts (Figure 1C and 1D). Median Dkk-3 levels did not significantly differ between healthy males (37.8 ng/ml, range 20.7 – 67.4 ng/ml) and healthy females (35.3 ng/ml, range 22.6 – 53.3 ng/ml), nor between male (28.6 ng/ml, range 15.4 – 62.3 ng/ml) and female (29.9 ng/ml, range 10.4 – 67.8 ng/ml) CRC patients (data not shown). Furthermore, no association was found between serum Dkk-3 levels and age in healthy controls (Spearman r = 0.182, p = 0.226), data not shown.

ROC curves were generated to quantify the ability of serum Dkk-3 levels to discriminate between healthy controls and those with CRC (AUC = 0.69; 95% CI 0.60-0.78; p = 0.0003) (Figure 2). At 90% specificity, serum Dkk-3 levels distinguished CRC patients with 36% sensitivity. Furthermore, at 90% specificity, serum Dkk-3 levels were discriminated between controls and patients with Dukes' stage A, B, C and D with a sensitivity of 24%, 32%, 47% and 38%, respectively.

Discussion

The Wnt pathway plays a context dependent role in a range of normal cellular processes, including proliferation, differentiation, and apoptosis [2]. In the gastrointestinal tract it plays a pivotal function in tissue homeostasis where patterning and organization of the crypt-villus axis is dependent on a gradient of Wnt signalling [28]. Reports examining the role of the Wnt antagonist Dkk-3 in CRC have demonstrated that Dkk-3 appears to have two divergent roles in colorectal carcinogenesis: one, as a tumour suppressor and two, as a pro-angiogenic factor in vascularisation. In vitro studies have demonstrated that Dkk-3 can reduce CRC cell line proliferation and induce apoptosis via activation of caspase-3 and caspase-9 [29]. Studies of primary CRC tumours have demonstrated reduced Dkk-3 expression in CRC tissues and that this silencing is primarily due to methylation of the DKK3 promoter [16,30,31]. Together, these findings suggest Dkk-3 plays a tumour suppressor role in colorectal carcinogenesis. In contrast, work by St Croix et al. [32] identified 46 tumour endothelial makers that demonstrated stronger gene expression in the endothelium of CRC tissues than in normal colonic endothelium. DKK3 was identified to be one of these genes; however, its role in tumour angiogenesis has not been resolved. Follow-up studies by other groups have confirmed this observation by demonstrating upregulation of Dkk-3 protein expression in the tumour endothelium of CRC and appears to be involved in angiogenesis [24,25].

Dkk-3 has a significant role in the modulation of the Wnt pathway and in colorectal carcinogenesis, and this is the first report to examine serum Dkk-3 protein levels in patients with CRC. It is widely accepted that the expression of DKK3 is frequently lost in human cancer tissue, and in this study we have found that patients with CRC had significantly (p = 0.0003) lower serum Dkk-3 protein levels than those of age- and gender-matched healthy controls. This finding is consistent with another report by Jiang et al who investigated circulating levels of DKK-3 in gynaecological cancers [33]. These authors observed lower

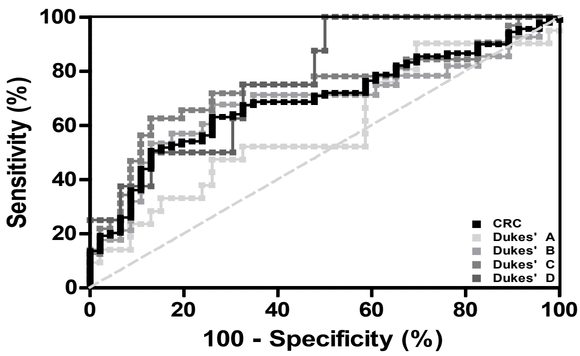

Figure 2: Receiver operating characteristic (ROC) curves for the ability of serum Dkk-3 levels to discriminate between healthy controls and patients with CRC (CRC). At 90% specificity, serum Dkk-3 levels distinguished CRC patients (all Dukes' stages) with 36% sensitivity. At 90% specificity, the sensitivity was 24%, 32%, 47% and 38%, respectively for Dukes' stages A, B, C, and D. Diagnostic subgroups are denoted as follows: CRC (encompassing all Dukes' stages) [■], Dukes' A [■], Dukes' B [■], Dukes' C [■], Dukes' D [■]. The line of identity is represented by a grey dashed line.

serum levels of DKK-3 in ovarian cancer patients when compared to normal individuals. In contrast, these authors also reported higher DKK-3 levels were found in cervical and endometrial cancers. Jiang et al. [33] also reported serum DKK-3 levels that were significantly higher (e.g., mean ± standard deviation of 42 ± 15 pg/mL in their normal patient group) than what is reported here (median and range, 36.8 ng/ml and 20.7– 67.4 ng/ml, respectively). Zenzmaier et al. [34] however, report DKK-3 plasma levels of 51.3 ± 20.3 ng/mL in their cohort of healthy individuals which is consistent with the measurements we have obtained [34]. These differences in the levels of circulating DKK-3 highlight the importance of inter-laboratory assay validation of candidate biomarkers when assessing their suitability for disease diagnosis.

Currently, colonoscopy and the faecal occult blood test (FOBT) are approved for clinical diagnosis of CRC. While colonoscopy has a sensitivity and specificity greater than 95% for CRC diagnosis, it is invasive and expensive. Conversely, the FOBT is a much cheaper alternative but has low sensitivity, especially for diagnosis of early stage disease [35,36]. Accordingly, identification of biomarker(s) that is specific for CRC and development of a non-invasive test would be beneficial. It is also widely accepted that a single biomarker when used alone will not provide adequate sensitivity and specificity for a diagnostic test, especially as CRC is a heterogeneous disease. Hence, a panel of biomarkers, that reflects this heterogeneity, will need to be identified. It is possible that DKK-3 may be suitable as one member of this panel.

DKK3 methylation status in tumour tissue has been widely examined as a prognostic indicator in a number of cancers where is has been shown to be a predictor of poor survival and shorter disease free survival in cervical cancer [26], endometrial cancer [37], hepatocellular carcinoma [23], gastric cancer [16], and breast cancer [22]. In a study by Yu et al. [16], DKK3 methylation was not found to be a good prognostic indicator of survival in a cohort of 84 CRC patients where CRC tissue was examined [16]. To the best of our knowledge, this is the only published report examining DKK3 and patient prognosis in CRC. We are currently follow-up information for these patients to determine the true potential of circulating Dkk-3 serum levels for identifying patients at high risk for disease recurrence.

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