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The prognostic significance of tumour FXYD-3 expression in patients with primary and secondary colorectal cancers

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Abstract

Purpose: *The clinical significance of FXYD-3 expression in colorectal cancer (CRC) is uncertain. There are no studies examining FXYD-3 expression in colorectal liver metastases (CRLM). The aim was to examine the degree of FXYD-3 expression in CRC and CRLM and assess the relationship between expression and survival after resection of liver metastases.*

Methods: *A matched case-control study, using conditional logistic regression, compared FXYD-3 expression in the primary tumours of patients without CRLM to those with CRLM. A retrospective cohort study on CRLM patients examined if FXYD-3 expression was associated with overall survival.*

Results: *There were 220 patients; 110 CRLM patients and 110 CRC patients. There was no difference in FXYD-3 expression between primary tumours (p=0.62) and no difference in FXYD-3 expression between the primary tumour and liver metastases (p=0.55). Strong FXYD-3 expression in CRLM's was associated with a reduced risk of mortality in comparison to weak FXYD-3 expression (HR: 0.51, 95% CI 0.28, 0.93). There was evidence of effect modification between FXYD-3 expression and chemotherapy (p<0.001): patients with strong FXYD-3 expression in CRLM's who received post-operative chemotherapy had longer survival compared to those who did not.*

Conclusion: *There are no differences in FXYD-3 expression in primary tumours between patients who did or did not develop liver metastases. Similarly, no difference in FXYD-3 expression between the primary tumour and the corresponding liver metastases. However, strong FXYD-3 expression in CRLM proved to be a marker of long-term survival in patients who receive adjuvant chemotherapy post liver resection.*

Keywords: *FXYD-3; colorectal liver metastasis; colorectal cancer; survival analyses*

Introduction

Colorectal cancer (CRC) is the third most common cancer in Australia with approximately 50% of patients eventually developing liver metastases (CRLM) [1,2]. Resection provides the best chance of both overall survival (OS) and disease-free survival (DFS). Neoadjuvant chemotherapy is often given to patients with either up-front resectable, or borderline resectable CRLM, aiming to downstage the size and/or the number of tumours [3,4]. Prognostic factors following liver resection include large tumours, multiple tumours, synchronous CRLMs, positive resection margins, extra-hepatic disease and K-RAS mutation status [5-7]. Although helpful, more accurate biomarkers are needed to facilitate selection of appropriate treatment as well as to determine prognosis [6,8]. Chemotherapy may cause debilitating side effects and specifically may lead to hepatotoxicity, which increases the risk of peri-operative complications if patients proceed to resection. In some patients this may offset any survival benefit [9]. Responses to chemotherapy vary and are likely to reflect the underlying biology of the tumour.

FXYD domain-containing ion transport regulator 3 (FXYD-3) regulates the activity of the sodium/potassium-transporting ATPase which transports Na+ out of the cell and K+ into the cell. Many organs such as the breast, pancreas, lung, stomach and rectum have varying expression levels of FXYD-3, but it is over-expressed in breast cancer [10], pancreatic ductal cancer [11], gastric cancer [12] and rectal cancer as well as some normal mucosal cells [13]. In some normal tissues such as the stomach, uterus and skin, FXYD-3 has a weak expression pattern [13]. MAT-8, a member of the FXYD family of proteins is upregulated by 5-FU in a p53-dependent manner [14] suggesting that FXYD-3 over-expression might promote metastases. This may be important because tumours harbouring mutated p53 genes are known to have a worse prognosis than patients without point mutations [15]. Loftas et al. [16] showed that strong expression of FXYD-3 may

be an indicator of reduced radio-sensitivity and therefore reduced survival, while Widegren et al. [13] found no correlation between FXYD-3 expression and survival. Loftas et al. [17] also found that FXYD-3 expression had no impact on survival of patients with recurrent CRC. Findings to date in CRC have been inconsistent. For example, Simmer et al. [18], showed that strong expression of FXYD-3 was associated with an increase in progression-free survival. Therefore, the exact role of the FXYD-3 protein in patients with primary CRC remains unclear. Wang [9] examined the prevalence of FXYD-3 in hepatocellular cancer (HCC) and concluded that strong expression of FXYD-3 correlated with reduced survival in HCC. To date there have been no studies of FXYD-3 expression in patients with CRLM.

We hypothesise that FXYD-3 expression may be an independent predictive factor in patients who undergo liver resection and, consequently this may help determine the role of post-resection chemotherapy. The aim of this study was to determine the prevalence of FXYD-3 expression in a series of patients with colorectal cancer; one group with primary CRC who did not develop CRLM on long-term follow-up and another group with primary CRC who developed liver metastases (CRLM patients). A second aim was to determine if there are differences in FXYD-3 expression between the primary tumours and the corresponding liver metastases, and to assess the impact of FXYD-3 on long-term survival.

Methods

Patients

Two groups of patients who underwent resection of CRC were studied. The first consisted of patients who had resection of CRC between 1998 and 2012 but who did not develop liver metastases on subsequent median follow of 5.5 years (NCRLM group). The second consisted of patients who underwent resection of a CRC between 1999 and 2012, and who also had either synchronous or metachronous CRLM resected with curative intent (CRLM group). Both groups of patients were identified from two different prospectively collected databases. A matched case-control study was conducted to compare the FXYD-3 expression between the NCRLM group patients (controls) and CRLM group patients (cases) using 1:1 matching. Matching was based on age (within a three-year difference), gender, stage and grade of the primary tumour. The sample of matched case-control design CRLM patients (cases) formed our retrospective cohort for determining if FXYD-3 expression was associated with overall survival.

Data concerning demographical patient information (age and sex), radiological investigations (timing, primary side, tumour size, stage of cancer), biochemical markers (apical node status, BRAF status, primary tumour grade, microsatellite instability (MSI) status, mismatch repair (MMR) status, tumour markers etc.), pre-operative treatments (radiotherapy, chemotherapy), intra-operative details (diameter of largest tumour, number of tumours), post-operative outcomes (complications, length of stay, survival, and post-operative treatment) from the CRLM patients was obtained from the prospectively collected database. Ethical approval for this study was obtained from the Northern Sydney Local Health District Human Research Ethics Committee (RESP/15/213) where consent was obtained for storing tumour bank tissues.

Immunohistochemical staining

RImmunohistochemical staining was performed using five-micrometre formalin-fixed paraffin-embedded slides. The slides were incubated at 60°Celsius for 12 hours, de-paraffinized and then rehydrated. To obtain the antigen, the slides were then transferred to 0.01 mol/L Tris-EDTA acid buffer (pH 9.0) and placed on a high-pressure container for 8 minutes. This was then incubated for a period of 30 minutes at room temperature followed by washing in PBS (pH 7.4). Another incubation period followed for 20 minutes with 3% H2O2 in methanol to block endogenous peroxidase activity. DAKO Protein Block serum free was used for 10 minutes to ensure non-specific antibody binding/staining. Once the blocking solution was removed, the slides were incubated in a moist chamber with a monoclonal anti-FXYD-3 primary antibody in 1:2 diluted in antibody diluent (DAKO) overnight at 4 degrees Celsius. These slides were then incubated with a goat anti-mouse/rabbit immunoglobulin for 25 minutes and were washed with PBS three times between each incubation step. The peroxidase reaction was performed for 8 minutes in 3,3 diaminobenzidine tetrahydrochloride (DAB) solution. The slides were rinsed with water, counterstained with Mayer's hematoxylin, and then washed, dehydrated in ethanol, and mounted with xylene-based mounting medium. The slides which were known to show strong immunostaining for FXYD-3 were used either receiving the primary antibody or the universal mouse IgG as positive and negative controls. All positive controls produced a clear stain while all negative controls showed no evidence of staining.

The slides were independently examined by two blinded investigators. In the event of a lack of agreement between the investigators the slides were re-stained followed by repeat examination by dual microscopy until an agreed score was reached. The intensity of FXYD-3 staining was scored as nil, low, moderate or high. The score was based on the maximum intensity of staining over most of the slide. After excluding for non-specific straining, the grading of intensity was scored relative to staining patterns across all slides. The most intensely stained slides were classified as "high". The monoclonal anti-FXYD-3 primary antibody was very specific with almost no background staining, and absence of staining relative to the positive and negative controls was classified as "nil" expression. Representative stains are shown in (Figure 1).

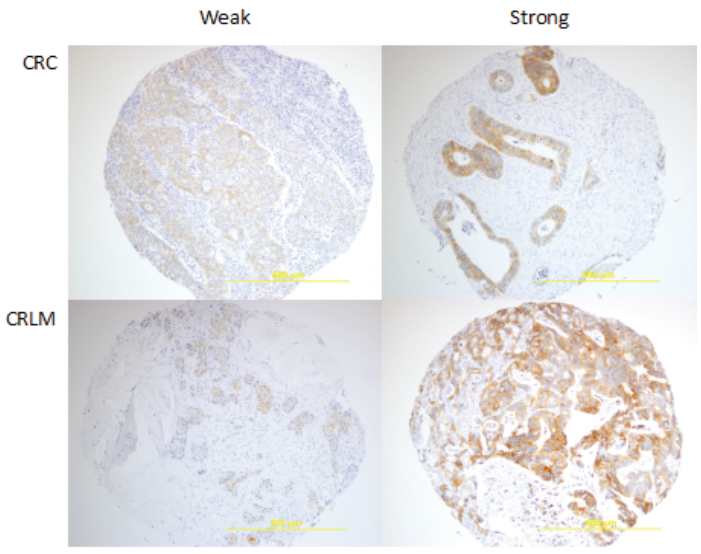

Figure 1: Representative TMA cores showing weak and strong FXYD-3 immunohistochemical staining, for both CRC and CRLM

Statistical analysis

FXYD-3 expression

The variable of interest was FXYD-3 expression. FXYD-3 expression slides that had a score of nil or low were grouped together as "weakly" stained and slides that had a moderate or high score were grouped together as "strongly" stained. For the CLRM patients, baseline characteristics were summarised according to weak and strong FXYD-3 expressions using frequencies and proportions. Differences in baseline characteristics between weak and strong FYXD-3 expressions were assessed using Pearson Chi-square or Fishers' exact tests.

Conditional logistic regression was used to examine the difference in FXYD-3 expression between the NCRLM patients and CRLM patients. Conditional logistic regression was used to adjust for the matching of controls to cases. Conditional logistic regression was also used to compare FXYD-3 expression in CRLM patients measured at the time of the primary resection and at liver resection.

Overall Survival in the sample of patients who developed CRLM

OS was assessed in patients who underwent resection of CRLM using time (in months) from liver resection to all-cause mortality. Kaplan-Meier curves were produced for overall survival by level of FXYD-3 expression in the liver metastasis. Univariable survival models were performed using the Cox proportional hazards model. All univariable variables that had a p-value < 0.3 were placed into the multivariable model. Variables with the largest p-value were removed one at a time from the multivariable model until we obtained a model that included FXYD-3 expression, significant predictors and confounders of FXYD-3 expression. A significance level of 0.05 was used for all analyses. A confounder was defined as a variable that changed the Hazard ratio of FXYD-3 expression by 20% or more once removed from the model when compared to when it was present in the model. Our final model consisted of Primary tumour and nodal stage, any recurrent disease (yes or no), diameter of largest liver tumour, complications after liver surgery (None/Minor vs. Major), SIRT treatment (yes or no) and FXYD-3 expression in the liver metastasis. To answer the question of whether chemotherapy was an effect modifier, post-operative chemotherapy was put back into the model and tested for an interaction with FXYD-3 expression in the liver metastasis. Due to the relatively small sample size of the whole study, it was not possible to assess the relationship between FXYD3 and treatment using biological agents. Similarly, the effects of different chemotherapy regimens and cycles were not examined due to the variability in administration in a small sample and for this reason a dichotomous variable was examined (yes or no). The proportional hazards assumption was verified after stratifying by recurrent disease using the Schoenfeld residuals and the overall goodness of fit of the model was assessed using Cox-Snell residuals. All statistical analysis was performed using Stata SE.

Due to inconsistent histopathological reporting, particularly in the early years of the study period, some data was missing in relation to the primary tumour histopathology in the sample of patients who developed CRLM. This included the following: MMR, MSI, pattern of growth, BRAF status, apical node status, tumour grade, tumour size, peri-tumour response and extramural venous permeation. Data in relation to the liver resection were missing in 12 patients. This included the duration of the operation (n=10), intra-operative blood loss (n=2), length of stay (n=1), and FXYD-3 expression (n=12). To deal with missing data, multiple imputations were used [19]. The chained equations method was used by imputing 10 data sets. Imputation was based on the following variables: age, sex, censor status, log of time to death/censored, timing of primary, primary side, primary T stage and N stage, operation type (laparoscopic or open), primary site, preoperative chemotherapy, tumour site of liver, diameter of largest liver tumour, number of liver tumours, type of liver resection, resection margin of liver, complications after liver surgery, inflow occlusion yes/no and post liver operative chemotherapy.

Results

The study sample consisted of 220 patients; 110 patients who underwent CRC resection who did not develop liver metastases (NCRLM group) and 110 patients who underwent CRC resection but subsequently developed liver metastases (CRLM group). FXYD-3 expressions were obtained for 108 out of 110 CRC patients (98%) and 98 out of 110 CRLM patients (89%).

Table 1 compares FXYD-3 expression between NCRLM patients (controls) and CRLM patients (cases). There was no difference in the staining patterns between the groups (p=0.62). The odds of low FXYD-3 expression compared to no expression in cases is 1.04 (95% CI 0.36, 3.01) compared to the odds of low vs. nil in the control group. Table 2 compares the FXYD-3 expression for the CRLM patients, measured at time of primary resection and measured again at time of liver resection. Again, there was no difference in the staining patterns at both time points (p=0.55).

Table 3 summarizes the patient characteristics classified by FXYD-3 expression in the liver metastases. FXYD-3 expression showed weak staining in 29 (29.6%) patients and strong staining in 69 (70.4%) patients. There was no significant difference in FXYD-3 expressions for sex or age (p>0.05). In primary tumours, there was no difference in FXYD-3 expression in relation to the timing of liver metastasis (synchronous or metachronous), neo-adjuvant chemotherapy, primary resection year, tumour location, tumour stage, MSI Status, MMR Status, BRAF status, pattern of tumour growth or the extramural venous response (p>0.05).

Weak FXYD-3 expression was associated with a higher frequency of distant nodal metastases (N2) than in strong FXYD-3 expression group (41.4% vs. 11.6%, p=0.003). In 81 patients who had a recorded apical node status, weaker FXYD-3 expression was more likely to be associated with nodal metastases than strong FXYD-3 expression (20% vs. 1.8%, p=0.009). Similarly, for the 71 patients with a recorded tumour grade, strong FXYD-3 expression was seen in a higher proportion of patients with a less well differentiated tumour grade (69.2% vs. 44.0%) and a lower proportion with a Well and Moderate tumour grade (30.8% vs. 56.0%) in comparison to patients with a weak FXYD-3 expression (p=0.03). In the liver metastases specimens, there was no difference in FXYD-3 expression for all the variables.

Table 4 shows the unadjusted and adjusted HR for FXYD-3 expression in the liver metastasis (see Appendix (Table A1) for all univariable analyses). After adjusting for the significant variables in the multivariable model, strong FXYD-3 expression in the liver metastasis was associated with a reduced hazard of death by 58% in comparison to patients with weak FXYD-3 expression in the liver metastasis.

Table 5 shows evidence that chemotherapy is an effect modifier with FYXD-3 expression in the liver metastasis: patients who have weak FXYD-3 expression and have been administered chemotherapy have a 14.80 times higher risk of death (HR=14.80 95% CI 1.69, 129.46) in comparison to those who did not receive chemotherapy following liver resection (p=<0.001). In contrast, patients with strong FXYD-3 expression who were administered chemotherapy post liver resection had a 48% lower risk of death (HR=0.52, 95% CI 0.22, 1.27) in comparison to those that did not receive chemotherapy (p=0.13) (see Appendix (Table A2) for

Table 1: *A comparison of FXYD-3 expression between cases and controls*

FXYD-3 expression in primary	Controls (n=107) n (%)	Cases (n=108) n (%)	Odds Ratio	95% CI	P-value
Nil	9 (8)	8 (8)	Reference		0.62
Low	24 (23)	23 (21)	1.04	(0.36, 3.01)	
Moderate	44 (41)	53 (49)	1.34	(0.48, 3.70)	
High	30 (28)	24 (22)	0.83	(0.28, 2.48)	

Table 2: *A comparison of FXYD-3 expression between primary CRC and liver metastasis*

FXYD-3 expression	Primary CRC (n=108) n (%)	Liver metastasis (n=98) n (%)	Odds Ratio	95% CI	P-value
Nil	8 (8)	12 (12)	Reference		0.55
Low	23 (21)	17 (17)	0.46	(0.15, 1.47)	
Moderate	53 (49)	46 (47)	0.54	(0.19, 1.54)	
High	24 (22)	23 (24)	0.71	(0.22, 2.32)	

Table 3: *Patient Characteristics classified according to FXYD-3 expression in the liver metastasis*

	Total Population (n=98)	Weak FXYD-3 expression (n=29)	Strong FXYD-3 expression (n=69)	P-value
Patient Characteristics				
Sex Male Female	 59 (60) 39 (40)	 18 (62) 11 (38)	 41 (59) 28 (41)	0.81
Age ≤55 56-65 66-70 ≥71	 21 (21) 26 (27) 22 (22) 29 (30)	 9 (31) 8 (28) 5 (17) 7 (24)	 12 (17) 18 (26) 17 (25) 22 (32)	0.46
Primary Disease				
Timing Synchronous Metachronous	 46 (47) 52 (53)	 13 (45) 16 (55)	 33 (48) 36 (52)	0.79
Neo-adjuvant Chemo Yes No	 4 (4) 94 (96)	 1 (3) 28 (97)	 3 (4) 66 (96)	0.99
CRC Primary Resection 1998-2006 2007-2014	 45 (46) 53 (54)	 15 (52) 14 (48)	 30 (43) 39 (57)	0.46
Primary Side Right Side Left Side	 31 (32) 67 (68)	 12 (41) 17 (59)	 19 (28) 50 (72)	0.18
Primary T stage T0, T1 or Tx, T2 T3 T4	 18 (18) 49 (50) 31 (32)	 5 (17) 14 (48) 10 (35)	 13 (19) 35 (51) 21 (30)	0.96
Primary N Stage N0 N1 N2	 35 (36) 43 (44) 20 (20)	 6 (21) 11 (38) 12 (41)	 29 (42) 32 (46) 8 (12)	0.003

	Total Population (n=98)	Weak FXYD-3 expression (n=29)	Strong FXYD-3 expression (n=69)	P-value
IHC MSI Status (n=81) High Low	4 (5) 77 (95)	3 (11) 24 (89)	1 (2) 53 (98)	0.11
Apical Node Status (n=82) Not involved Positive for tumour	76 (93) 6 (7)	20 (80) 5 (20)	56 (98) 1 (2)	0.009
Tumour Grade (n=77) Poor Well and Moderate	47 (61) 30 (39)	11 (44) 14 (56)	36 (69) 16 (31)	0.03
BRAF Status (n=81) Positive Negative	7 (9) 74 (91)	2 (7) 25 (93)	5 (9) 49 (91)	0.99
MMR Status (n=80) MMR Deficient MMR Proficient	4 (5) 76 (95)	3 (11) 24 (89)	1 (2) 52 (98)	0.11
Pattern of Growth (n=61) Infiltrative Non-Infiltrative	36 (59) 25 (41)	9 (64) 5 (36)	27 (57) 20 (43)	0.76
Extramural venous permeation (n=59) Present Absent	20 (34) 39 (66)	4 (29) 10 (71)	16 (36) 29 (64)	0.75
Peritumour Lymph response (n=59) Present Absent	32 (54) 27 (46)	7 (50) 7 (50)	25 (56) 20 (44)	0.72
Liver Metastases				
CRLM resection year 1998-2006 2007-2014	38 (39) 60 (61)	15 (52) 14 (48)	23 (33) 46 (67)	0.09
Serum tumour markers Elevated Not Elevated	75 (77) 23 (23)	22 (76) 7 (24)	53 (77) 16 (23)	0.99
Pre-Op Chemotherapy Yes No	70 (71) 28 (29)	24 (83) 5 (17)	46 (67) 23 (33)	0.14
Tumour site Unilateral Bilateral/Central	74 (76) 24 (24)	22 (76) 7 (24)	52 (75) 17 (25)	0.96
Diameter of largest tumour (mm) <20 20≤ Diameter (mm)<30 30≤ Diameter (mm)<55 ≥55	16 (16) 27 (28) 29 (29) 26 (27)	6 (21) 8 (28) 10 (34) 5 (17)	10 (14) 19 (28) 19 (28) 21 (30)	0.54
Number of tumours Single Multiple	52 (53) 46 (47)	16 (55) 13 (45)	36 (52) 33 (48)	0.79
Resection Margin R0 R1	87 (89) 11 (11)	28 (97) 1 (3)	59 (86) 10 (14)	0.17
Post-operative comps None/Minor Major	82 (84) 16 (16)	24 (83) 5 (17)	58 (84) 11 (16)	0.99
Resection Detail Segmentectomy Sectionectomy Hemi-hepatectomy Extended hemi-hepatectomy Wedge	28 (29) 20 (20) 25 (26) 12 (12) 13 (13)	6 (21) 7 (24) 8 (27) 2 (7) 6 (21)	22 (32) 13 (19) 17 (25) 10 (14) 7 (10)	0.43

	Total Population (n=98)	Weak FXYD-3 expression (n=29)	Strong FXYD-3 expression (n=69)	P-value
Tumour Differentiation				
Well	7 (7)	1 (4)	6 (9)	0.42
Moderate	74 (76)	21 (72)	53 (77)	
Poor	5 (5)	3 (10)	2 (3)	
N/A	12 (12)	4 (14)	8 (11)	
Post-operative Radiotherapy				
Yes	6 (6.1)	1 (3.5)	5 (7.3)	0.67
No	92 (93.9)	28 (96.5)	64 (92.7)	
Post-operative SIR-Spheres				
Yes	9 (9)	4 (14)	5 (7)	0.44
No	89 (91)	25 (86)	64 (93)	
Post-operative Chemotherapy				
Yes	67 (68)	24 (83)	43 (62)	0.06
No	31 (32)	5 (17)	26 (38)	
Recurrent disease				
Yes	70 (71)	22 (76)	48 (70)	0.63
No	28 (29)	7 (24)	21 (30)	
Multiple resections				
Yes	14 (14)	2 (7)	12 (17)	0.22
No	84 (86)	27 (93)	57 (83)	

Table 4: *Hazard ratios (HR) for FXYD-3 Expression in the liver metastasis on overall survival for unadjusted and adjusted models*

HR (95% CI)	Weak FXYD-3 Expression	Strong FXYD-3 Expression	P-value
Unadjusted	1	0.51 (0.28, 0.93)	0.03
Adjusted* HR (95% CI)	1	0.42 (0.21, 0.87)	0.02

* Adjusted for primary T and N stage, diameter of largest liver tumour, post liver operative complications, recurrent disease and post liver operative SIR-Spheres

Table 5: *Hazard ratios (HR) for FXYD-3 Expression in the liver metastasis on overall survival with chemotherapy*

FXYD-3 expression	No Chemotherapy**	Chemotherapy**	P-value	P-value of interaction
Weak	1	14.80 (1.69, 129.46)	0.015	<0.001
Strong	1	0.52 (0.22, 1.27)	0.125	

** Adjusted for primary T and N stage, diameter of largest liver tumour, post liver operative complications, post liver operative SIR-Spheres and chemotherapy. Stratified by recurrent disease.

full model). The Cox-Snell plots indicated that the multivariable model(s) fitted the data well and there was no violation of the Proportional Hazards assumption.

Discussion

This study found no association between expression of FXYD-3 in primary colorectal cancers and the risk of developing liver metastases. Furthermore, there was no concordance of expression between primary colorectal cancers and the corresponding liver metastases. However, both univariable and multivariable analyses suggest that FXYD-3 expression in liver metastases is an independent predictor of survival in CRLM patients. Specifically, strong FXYD-3 expression in liver metastases, conferred a survival benefit compared to weak expression. The results were consistent even after adjusting for the primary T and N stage, the diameter of the largest liver tumour, post liver resection operative complications, recurrent disease and SIR-sphere treatment.

Amongst patients with strong FXYD-3 expression, patients who had adjuvant chemo had better survival than those that did not. Patients who had weak FXYD-3 expression and received adjuvant chemotherapy had the poorest prognosis. This observation may be in part explained by the fact that most of the chemotherapy was 5FU-based, and weak expression of FXYD-3 may be acting as a surrogate marker of an unfavourable p53 status [13].

The clinical relevance of FXYD-3 expression in colorectal tumours is evolving. Widegren et al. [13], concluded that there was no significant relationship between FXYD-3 expression and CRC survival. However, this was despite finding that patients with node-negative disease (Dukes A or Dukes B stage) and strong primary tumour FXYD-3 expression had worse long-term survival than patients weak FXYD-3 expression. Similarly, Loftas et al. [17] found that FXYD-3 expression did not correlate with survival in patients with recurrent CRC. However, Loftas et al. [17] defined a weak FXYD-3 expression as either nil, low or moderate. In the

present study, and in the one conducted by Widegren et al. [13], weak FXYD-3 expression was defined as either nil or low only. Even after altering the definitions of weak and strong FXYD-3 expression, Loftas et al. [17] found similar outcomes. Loftas et al. [16] suggested that strong expression of FXYD-3 reduces the effects of radiotherapy and hence reduces survival in CRC patients. Simmer et al. [18], found that strong expression of FXYD-3 was associated with an increase in progression-free survival. Wang et al. [19] showed that strong tumour expression of FXYD-3 was associated with reduced survival in patients with hepatocellular cancer (HCC) compared to patients with weak FXYD-3 expression. Until now no other study has examined the relationship between FXYD-3 expression in tumour tissue and survival after resection of colorectal liver metastases.

The limitations of this study include the relatively small data set, consisting of only 110 patients, and the small number of missing data variables due to inconsistencies in some of the pathology reports. The missing variables were overcome by performing multiple imputations, and the final model consisted of variables that only had a few missing observations. Furthermore, a small sample size prevented the analysis of specific chemotherapy and immunotherapy regimens, however most patients (~70%) who received adjuvant therapy were 5FU-based.

In the final model, it was not possible to make definitive conclusions about the significance of FXYD-3 expression in the primary tumour cohort without CRLM because there were only two deaths (2%) after a median follow up of 5.6 years. In contrast, patients with strong expression of FXYD-3 in the CRLM had better long-term survival compared to patients with weak expression. The reasons for this are unclear but may be because many of these patients had received prolonged systemic chemotherapy prior to undergoing liver resection. Further work and with larger patient cohorts is required to determine whether other factors such as gene mutation status (e.g. KRAS, BRAF) might be responsible for these findings.

Conclusion

In conclusion, the present study found that there are no differences in FXYD-3 expression in primary tumours between patients who did or did not develop liver metastases. Similarly, there was no difference in FXYD-3 expression between the primary tumour and the corresponding liver metastases. In patients who received adjuvant chemotherapy post liver resection strong FXYD-3 expression in the CRLM was a marker of long-term survival. The significance of these findings are yet to be elucidated and further work is required to fully understand the role of FXYD-3 in the diagnosis and management of patients with colorectal cancer. Specifically, it will be important to examine the role of FXYD-3 in larger patient cohorts and whether there is any relationship with known important prognostic factors such as KRAS mutation status.

Declarations

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Conflicts of Interest/Competing interests: No conflicts of interest or competing interests declared

Ethics approval: Ethical approval for this study was obtained from the Northern Sydney Local Health District Human Research Ethics Committee (RESP/15/213) where consent was obtained for storing tumour bank tissues.

Consent to participate: All authors consented to participate

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Appendix

Table A1: Univariable results for overall survival

Variable	Hazard Ratio	95% CI		P-value
Patient Characteristics				
Sex Female	0.67	0.35	1.25	0.21
Age ≥71	1.7	0.9	3.21	0.11
Primary Disease				
Timing Metachronous	0.68	0.38	1.24	0.21
CR resection year 2007-2014	0.92	0.5	1.68	0.79
Primary Side Left sided	0.85	0.48	1.58	0.61
Primary T stage T3 T4	 1.24 2.65	 0.5 1.01	 3.08 6.7	0.03
Primary N Stage N1 N2	 1.06 2.69	 0.52 1.28	 2.19 5.66	0.01
IHC MSI Status High	1.59	0.46	5.47	0.46
Apical Node Status (n=92) Positive for tumour	1.82	0.76	4.38	0.18
Tumour Grade Well and Moderate	1.03	0.55	1.94	0.92
BRAF Status (n=91) Positive	1.68	0.6	4.71	0.33
MMR Status (n=90) Proficient	0.37	0.11	1.2	0.1
Pattern of Growth (n=71) Infiltrative	1.56	0.65	3.73	0.32
Extramural Venous Permeation Present	1.37	0.66	2.88	0.4
Peritumour Lymph response Present	0.85	0.4	1.79	0.66
Liver Metastases				
CRLM Surgery Year 2007 - 2014	1.11	0.61	2.04	0.73
Serum markers Elevated	1.65	0.79	3.43	0.18
Pre-Op Chemotherapy Yes	1.34	0.66	2.71	0.41
Tumour Site Bilateral/Central	1.09	0.56	2.11	0.8
Diameter of largest tumour (mm) 20≤ Diameter (mm)<30 30≤ Diameter (mm)<55 ≥55	 0.67 1.57 1.44	 0.27 0.71 0.61	 1.66 3.51 3.4	0.19

Number of tumours Multiple	1.24	0.69	2.22	0.48
Resection Margin R1	2.11	0.98	4.54	0.06
Post-operative Complications Major	1.68	0.78	3.62	0.19
Length of stay (days) 7-8 days ≥9 days	1.37 1.95	0.62 0.88	3.05 4.36	0.24
Resection Detail Sectionectomy Hemi-hepatectomy Extended hemi-hepatectomy Wedge	1.76 1.19 3.64 1.89	0.72 0.47 1.44 0.73	4.34 3 9.17 4.92	0.06
Tumour differentiation Moderate Poor N/A	1.3 2.65 1.17	0.4 0.53 0.29	4.25 13.21 4.69	0.63
FXYD-3 expression Moderate or High	0.51	0.28	0.93	0.03
Post-Operative Radiotherapy Yes	0.42	0.06	3.06	0.39
Post-Operative RFA Yes	1.08	0.26	4.48	0.91
Post-Operative SIR Spheres Yes	2.9	1.39	6.07	0.005
Post-Operative Chemotherapy Yes	1.68	0.85	3.33	0.14
Recurrent Disease Yes	6.89	2.46	19.32	<0.001
Multiple Liver resections Yes	0.6	0.24	1.52	0.28

Table A2: *Multivariable analysis for overall survival incorporating the effects of chemotherapy stratified by recurrent disease*

	Hazard Ratio	**95% CI**		**P-value**
Primary T stage T3 T4	0.74 3.92	0.27 1.42	2 10.79	<0.001
Primary N Stage N1 N2	1.21 2.94	0.52 1.17	2.83 7.4	0.03
Diameter of largest tumour (mm) 20≤ Diameter (mm)<30 30≤ Diameter (mm)<55 ≥55	1.06 2.18 3.8	0.35 0.87 1.25	3.21 5.49 11.58	0.03
Post-operative Complications Major	2.78	1.05	7.38	0.04
Post-Operative SIR Spheres Yes	3.57	1.32	9.67	0.01
FXYD-3 expression with Post op chemo-therapy Weak FXYD-3 and chemotherapy Strong FXYD-3 and no chemotherapy Strong FXYD-3 and chemotherapy	14.8 6.71 3.52	1.69 0.74 0.4	129.46 60.78 30.75	<0.001