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Membrane Proteins as Potential Colon Cancer Biomarkers: Verification of 4 Candidates from a Secretome Dataset

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Abstract

Colorectal cancer (CRC) is an important health issue in Taiwan. There were over ten thousand newly diagnosed CRC patients each year. The outcome of late stage CRC still remains to be improved, and tumor markers are expected to improve CRC detection and management. From a colorectal cancer cell secretome database, we chose four proteins as candidates for clinical verification, including tumor-associated calcium signal transducer 2 (TROP2, TACSTD2), transmembrane 9 superfamily member 2 (TM9SF2), and tetraspanin-6 (TSPAN6), and tumor necrosis factor receptor superfamily member 16 (NGFR). Different groups of 30 CRC patients' tissue samples collected from Chang Gung Memorial Hospital were analyzed by immunohistochemistry (IHC) for the four proteins, and the results were scored by pathologist. For all the four candidate proteins, marked differences of IHC score existed between tumor and adjacent non-tumor counterpart. However, there were only trends between higher protein expression levels and worse outcome. Three proteins (TROP2, TM9SF2 and NGFR) had trends between higher tissue expression and tumor stage or lymph node metastasis. Our study revealed that tissue expression of four proteins (TROP2, TM9SF2, TSPAN6, and NGFR) was markedly different between tumor and adjacent non-tumor counterparts. Overexpression of all these four proteins showed some trends with poorer survival.

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Keywords

Biomarker, Colorectal Cancer, Immunohistochemistry, Membrane Protein, Secretome, Tetraspanin-6, Transmembrane 9 Superfamily Member 2, Tumor-Associated Calcium Signal Transducer 2, Tumor Necrosis Factor Receptor Superfamily Member 16, Verification

1. Introduction

In Taiwan, colorectal cancer (CRC) has become an important health issue in recent years. There were more and more newly diagnosed CRC every year. In 2011, there were 14,087 CRC patients diagnosed in Taiwan [1]. Among them, 49.5% were diagnosed as late stage (stage III, 26.6%; stage IV, 22.8%). The five-year survival rate for late staged CRC was still relatively low (for colon cancer, stage III, 47.8%; stage IV, 10.3%) [2]. Tumor biomarkers were expected to integrate into CRC management to improve earlier diagnosis rate, to improve risk stratification, and to predict treatment response [3]-[7]. However, carcinoembryonic antigen (CEA), the most common serum biomarker currently used in clinic for CRC, doesn't meet all these clinical needs [8]-[12].

Although serum biomarkers play important role in cancer management, histology-based approaches are still the gold standard for tumor staging at present. Molecular staging provides an opportunity to personalized medicine and to maximize cost-effective management [13]-[16]. More and more analyses suggest that molecular approaches offer an advantage for stratifying patients further. Recently, some prospective studies have begun to predict risk of disease recurrence by molecular markers [17] [18]. For example, epidermal growth factor receptor (EGFR), which participates in signaling pathways that are deregulated in cancer cells and up-regulated in 50% - 80% CRC cases [19]-[24], has shown to be related to prognosis and therapeutic response of CRC patients [25] [26]. Many studies reported that Cetuximab, an antibody against EGFR, has additional benefit on CRC patients receiving chemotherapy [27] [28].

In recent years, the secretome-based approach has been proven to be a promising strategy for discovery of CRC biomarkers [29]-[31]. Using this approach, we have previously established a secretome dataset from 23 cancer cell lines, from which over one hundred proteins were selected identified from two CRC cell lines [29]. Among them, we had selected four protein candidates for verification, including tumor-associated calcium signal transducer 2 (TROP2, TACSTD2), transmembrane 9 superfamily member 2 (TM9SF2), tetraspanin-6 (TSPAN6), and tumor necrosis factor receptor superfamily member 16 (NGFR). These four proteins have been preliminarily examined in some CRC tissues in the Human Protein Atlas (HPA). Furthermore, there have been some reports about expression levels of TROP2 in different cancers including pancreatic cancer, CRC and ovarian carcinoma [32]-[35]. Tumor necrosis factor receptor superfamily member 16 (NGFR) has been studied in neurologic malignancy, which is also involved in cell growth control [36]-[39]. In functional aspect, TSPAN6 was found to be involved in cell motility [40] [41]. which may be related to tumor cell migration. We verified these four proteins in CRC tissues and their adjacent non-tumor counterparts by immunohistochemistry. We also analyzed the relationships between protein expression levels and clinicopathological factors of CRC patients.

2. Material & Methods

2.1. Patient Population and Clinical Specimen

All clinical samples were collected at Chang Gung Memorial Hospital (Taoyuan, Taiwan). Tissue samples were collected in 1995. Different groups of 30 CRC patients' tissue samples were used for our candidates. All CRC patients had histologically verified adenocarcinoma. All were subjected to a follow-up strategy that included regular outpatient visits, CEA test, and image studies. Patients characteristics, including gender, age, tumor location, histological grade, tumor stage, CEA level, operation date, tumor recurrence, follow up date, and follow up status, were obtained from clinical and pathology records. The study was approved by the Institutional Review Board at Chang Gung Memorial Hospital (IRB No. 99-0515B, 101-0712B and 102-1446C).

2.2. Immunohistochemistry

The tumor tissue blocks used for IHC were first fixed in 4% paraformaldehyde and then embedded in paraffin.

Sections (5 μm thick) were cut from tissue blocks, mounted on silanized slides (Superfrost, Menzel, Braunschweig, Germany), subsequently deparaffinized with xylene (twice for 10 min each), and rehydrated through ethanol gradient washes. Endogenous peroxidase activities are inactivated in 3% H_2O_2 before heating in a microwave oven for antigen retrieval (10 mm citrate buffer, pH 6.0; 20 min, 700 W). To block nonspecific binding, slides were preincubated with 10% nonimmune goat serum at 37˚C for 30 min. Slides were then incubated with anti-human primary antibody for 30 min at room temperature. Following washing with PBS (pH 7.4), slides were incubated with HRP-conjugated IgG secondary antibody for 30 min at room temperature and then developed using 3,3'-diaminobenzidine (Sigma, St Louis, MO). All procedure followed the standard pro-tocol. Expression of these protein was categorized as positive or negative and was evaluated according to the percentage of cells stained (0% - 100%) and the intensity of cell staining (3, strong; 2, moderate; 1, weak; or 0, no cell staining).

2.3. Statistical Analysis

For the analysis of IHC results, independent t test was used. The associations between protein expression and clinicopathological characteristics were analyzed using chi-square method and ANOVA. To determine factors related to overall survival, the probability was calculated using the Log-rank test by the Kaplan-Meier method. Cox proportional hazard models were used for maltivariate analysis. Comparative analysis of IHC scoring and CEA in paired CRC patients were undertaken. Statistical significance was set at $p < 0.05$. All analyses were performed using the statistical software, Statistical Package for the Social Sciences (Version 17.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Clinicopathological Analysis between High and Low Protein Expression Groups

All patients of each studied group for four candidate were divided into high expression group and low expression group. We used medium IHC scoring as cut-off value of high and low expression. It were 100 for TROP2, 110 for TM9SF2, 150 for TSPAN6, 110 for NGFR. We analyzed most clinicopathologic factors, including ages, gender, tumor location, histological grade, tumor stage, T stage, N stage, CEA level, and survival. It seems no differences between high and low expression groups for each protein (**Table 1**).

3.2. IHC Stain Scoring of Candidate Proteins between Tumor Tissues and Their Adjacent Non-Tumor Counterparts

For all four candidate proteins, IHC scoring between adjacent non-tumor area (AN) and tumor area (T) was much different. For TROP2, it was 3.33 ± 6.53 vs. 92.67 ± 22.06 (AN vs. T, $p < 0.01$). For TM9SF2, it was 7.66 ± 7.90 vs. 123.70 ± 22.05 (AN vs. T, $p < 0.01$). For TSPAN6, it was 3.33 ± 6.53 vs. 145.30 ± 17.97 (AN vs. T, $p < 0.01$). For NGFR, it was 6.00 ± 5.44 vs. 100.70 ± 12.60 (AN vs. T, $p < 0.01$) (**Figure 1**).

3.3. Comparison of IHC Stained Area and Staining Scoring According to Different Clinicopathological Factors

Furthermore, we compared mean proteins expression area and IHC scoring between different clinicopathologic factors. Although most factors had no statistical differences, patients with worse 5-year survival had trends of higher proteins expression area and IHC scoring. Patients with late tumor stage or positive lymph node metastasis had trends of higher protein expression (for TROP2, TM9SF2, NGFR). The trends also existed in histological grade (for TROP2 and NGFR) (**Table 2**).

3.4. Kaplan-Meier Survival Analysis According to High and Low Protein Expression

For all four proteins, high expression groups has the trend of worse survival, especial at 10-year. For TROP2, the 10-year survival rate of high expression and low expression groups were 28.5% vs. 50.0% ($p = 0.43$). For TM9SF2, the 10-year survival rate of high expression and low expression groups were18.0% vs. 42.0% ($p = 0.14$). For TSPAN6, the 10-year survival rate of high expression and low expression groups were 22.0% vs. 33.0% ($p = 0.60$). For NGFR, the 10-year survival rate of high expression and low expression groups were 52.9% vs.

Table 1. Analysis of clinicopathologic factors of different proteins expression groups.

	TROP2			TM9SF2			TSPAN6			NGFR		
	Low	High	p	Low	High	p	Low	High	p	Low	High	p
Gender												
Male	10 (62.5)	5 (35.7)	0.27	7 (50.0)	9 (56.3)	1.00	7 (58.3)	12 (66.7)	0.71	7 (53.8)	9 (52.9)	1.00
Female	6 (37.5)	9 (64.3)		7 (50.0)	7 (43.8)		5 (41.7)	6 (33.3)		6 (46.2)	8 (47.1)	
Age												
<65	5 (31.3)	4 (28.6)	1.00	6 (42.9)	7 (43.8)	1.00	6 (50.0)	10 (55.6)	1.00	7 (53.8)	11 (64.7)	0.54
≥65	11 (68.8)	10 (71.4)		8 (57.1)	9 (56.3)		6 (50.0)	8 (44.4)		6 (46.2)	6 (35.3)	
Location												
Colon	6 (37.5)	4 (28.6)		5 (35.7)	6 (37.5)		3 (25.0)	5 (27.8)		7 (53.8)	9 (52.9)	
Rectum	6 (37.5)	7 (50.0)	0.41	7 (50.0)	9 (56.3)	0.83	9 (75.0)	9 (50.0)	0.46	5 (38.5)	5 (29.4)	0.57
Other	4 (25.0)	3 (21.4)		2 (14.3)	1 (6.2)		0 (0)	4 (22.2)		1 (7.7)	3 (17.7)	
Histological grade												
WD	2 (12.5)	2 (14.3)		5 (35.7)	3 (18.8)		4 (33.3)	5 (27.8)		4 (30.8)	4 (23.5)	
MD	14 (87.5)	9 (64.3)	0.37	7 (50.0)	13 (81.2)	0.12	7 (58.3)	13 (72.2)	0.53	9 (69.2)	11 (64.7)	0.60
PD	0 (0)	1 (7.1)		2 (14.3)	0 (0)		1 (8.4)	0 (0)		0 (0)	2 (11.8)	
Stage												
Early	6 (37.5)	6 (42.9)	1.00	8 (57.1)	8 (50.0)	0.73	3 (25.0)	6 (33.3)	0.70	9 (69.2)	12 (70.6)	1.00
Late	10 (62.5)	8 (57.1)		6 (42.9)	8 (50.0)		9 (75.0)	12 (66.7)		4 (30.8)	5 (29.4)	
T stage												
T1-T2	6 (37.5)	6 (42.9)	1.00	3 (21.4)	2 (13.3)	0.56	2 (20.0)	2 (14.3)	1.00	1 (7.7)	3 (18.8)	0.60
T3-T4	10 (62.5)	8 (57.1)		11 (78.6)	13 (86.7)		8 (80.0)	12 (85.8)		12 (92.3)	13 (81.3)	
Lymph node metastasis												
Negative	9 (56.2)	8 (57.1)	1.00	8 (57.1)	8 (50.0)	0.69	3 (25.0)	9 (50.0)	0.26	9 (69.2)	12 (70.6)	1.00
Positive	7 (43.8)	6 (42.9)		6 (42.9)	8 (50.0)		9 (75.0)	9 (50.0)		4 (30.8)	5 (29.4)	
CEA												
<5	10 (62.5)	6 (42.9)	0.46	9 (64.3)	6 (37.5)	0.27	6 (50.0)	11 (61.1)	0.71	7 (53.8)	7 (41.2)	0.83
≥5	6 (37.5)	8 (57.1)		5 (35.7)	9 (56.3)		6 (50.0)	7 (38.9)		6 (46.2)	9 (52.9)	
5-year survival												
<5	6 (37.5)	6 (42.9)	1.00	6 (42.9)	10 (62.5)	0.46	6 (50.0)	11 (61.1)	0.71	4 (30.8)	8 (47.1)	0.36
≥5	10 (62.5)	8 (57.1)		8 (57.1)	6 (37.5)		6 (50.0)	7 (38.9)		9 (69.2)	9 (52.9)	

61.5% (p = 0.62) (**Figure 2**).

3.5. Multivariate Analysis

Using 5-year survival as end point, we further did multivariate analysis for all four proteins (**Table 3**). However, TROP2 and NGFR didn't show any differences. High TM9SF2 expression group had HR 1.22 (p = 0.72). High TSPAN6 expression group had HR 3.75 (p = 0.02).

3.6. Comparison Analysis with CEA

All four proteins tissue expression seemed not to be related to CEA level. However, all candidates increased

Table 2. Comparison of protein IHC scaring according to different clinicopathologic factors.

	TROP2				TM9SF2				TSPAN6				NGFR			
	Area (%)	p	Score	p	Area (%)	p	Score	p	Area (%)	p	Score	p	Area (%)	p	Score	p
Gender																
Male	65.3	0.44	82.0	0.26	83.1	0.23	125.6	0.26	86.3	0.10	144.7	0.93	73.7	0.31	98.7	0.75
Female	67.3		103.3		77.8		121.4		93.6		146.3		80.7		102.8	
Age																
<65	66.6	0.36	103.3	0.15	82.3	0.86	131.5	0.19	85.6	0.24	141.8	0.69	80.0	0.29	106.1	0.30
≥65	66.1		88.1		79.4		117.6		92.8		149.2		72.5		92.5	
Location																
Colon	58.0	0.21	91.0	0.48	85.4	0.31	140.9	0.35	86.2	0.62	138.7	0.36	76.8	0.89	100.0	0.80
Rectum	75.3		97.6		79.3		116.2		87.7		138.8		78.0		102.0	
Histological grade																
WD	67.5		92.5		80.0		110.0		88.8		148.8		75.0		100.0	
MD	61.7	0.42	83.4	0.35	79.5	0.71	132.0	0.56	89.5	0.87	145.0	0.86	76.5	0.93	99.0	0.73
PD	100.0		170.0		95.0		95.0		80.0		120.0		90.0		120.0	
Stage																
Early	60.8	0.43	85.0	0.58	76.8	0.38	111.8	0.28	94.4	0.11	150.0	0.74	75.2	0.44	99.0	0.70
Late	70.0		97.7		85.0		137.1		86.6		143.3		81.1		104.4	
T stage																
T1-T2	80.0	0.53	115.0	0.60	86.0	0.59	122.0	0.95	90.0	0.91	135.0	0.67	85.0	0.34	120.0	0.22
T3-T4	65.3		91.0		79.1		123.7		89.0		147.0		75.2		96.4	
Lymph node metastasis																
Negative	58.8	0.11	88.2	0.66	76.8	0.38	111.8	0.27	95.8	0.04	159.1	0.22	75.2	0.44	99.0	0.70
Positive	76.1		98.4		85.0		137.1		84.4		136.1		81.1		104.4	
CEA																
<5	61.8	0.41	81.1	0.38	77.3	0.51	112.6	0.36	89.4	0.93	148.8	0.67	72.8	0.35	95.0	0.50
≥5	71.4		103.5		83.5		134.2		88.4		140.7		79.3		104.0	
5-year survival																
<5	71.6	0.45	106.6	0.31	85.0	0.31	140.0	0.12	91.7	0.33	154.7	0.25	78.3	0.75	105.8	0.52
≥5	62.7		83.3		75.7		105.0		85.3		133.0		76.1		97.2	

detection of normal CEA cases. We used medium IHC scoring as cut off for each protein. They were 100 for TROP2, 110 for TM9SF2, 150 for TSPAN6, and 110 for NGFR, respectively. For TROP2, 6 among 16 normal CEA cases had higher TROP2 tissue expression. For TM9SF2, 6 among 15 normal CEA cases had higher tissue expression. For TSPAN6, 11 among 17 normal CEA cases had higher tissue expression. For NGFR, 7 among 14 normal CEA cases had higher tissue expression (**Figure 3**). All four proteins had the potential to improve false negative rate of CEA.

3.7. Expression Analysis among Stages with Normal and Abnormal CEA Levels

We further analyzed the percentage of high and low expression among early stage and late stage cases. We found that, for TROP2 and TM9SF2, late stage with abnormal CEA had high tissue protein expression, compared to early stage with normal. But it seemed not to be different for NGFR, and even to have reverse association

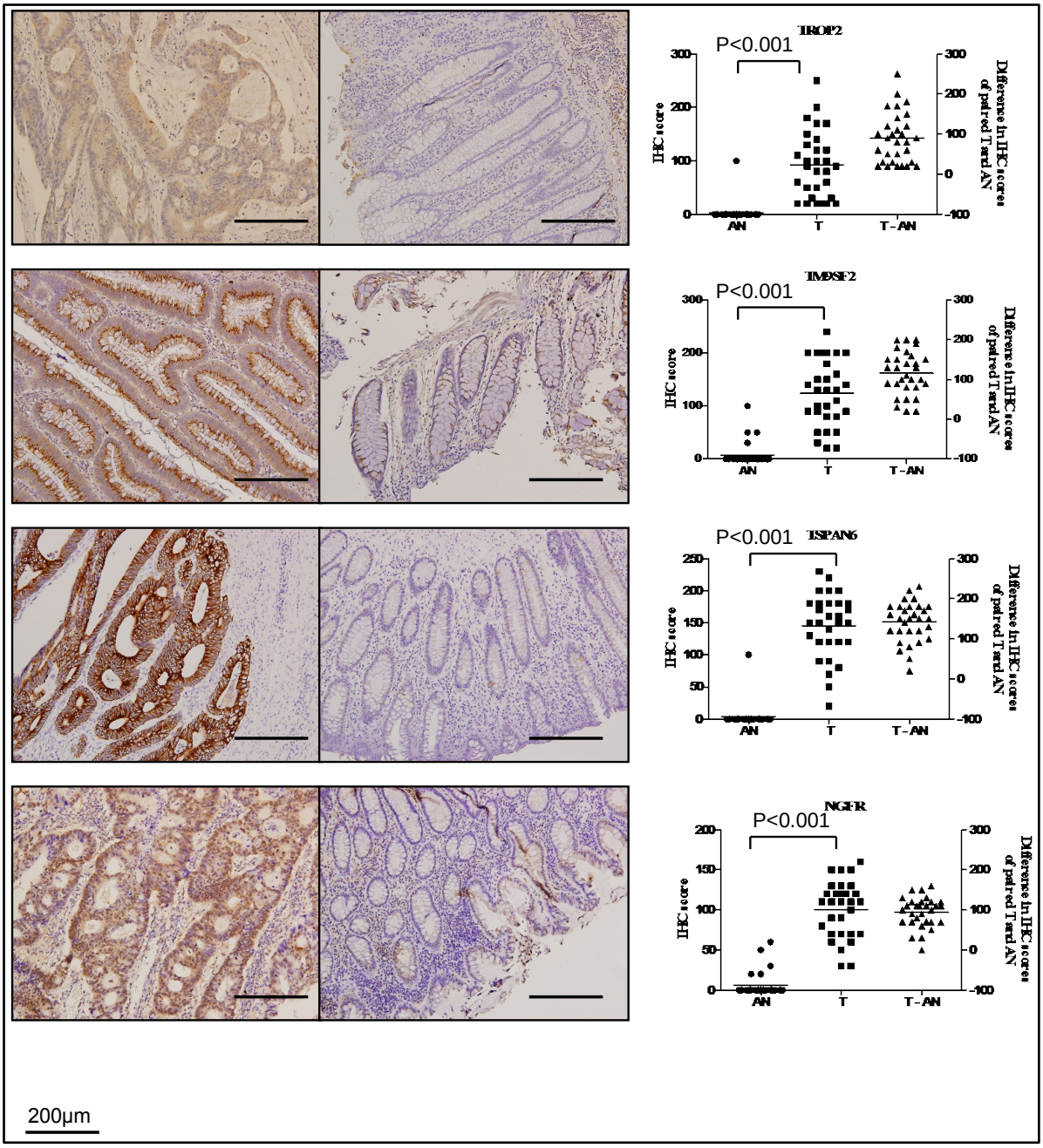


Figure 1. Immunohistochemical staining of TROP2 (A), TM9SF2 (B), TSPAN6 (C), and NGFR (D) in paired tumor (T) and adjuvant non-tumor (AN) tissues from different groups of 30 paired CRC patients (scale bar =200 μm). All these four proteins were expressed mainly in cytosol of tumor cells (A)-(D). The IHC scores were markedly different between tumor and adjuvant non-tumor tissues. For TROP2, it was 3.33 ± 6.53 vs. 92.67 ± 22.06 (AN vs. T, $p < 0.01$) (E). For TM9SF2, it was 7.66 ± 7.90 vs. 123.70 ± 22.05 (AN vs. T, $p < 0.01$) (F). For TSPAN6, it was 3.33 ± 6.53 vs. 145.30 ± 17.97 (AN vs. T, $p < 0.01$) (G). For NGFR, it was 6.00 ± 5.44 vs. 100.70 ± 12.60 (AN vs. T, $p < 0.01$) (H).

for TSPAN6 (**Figure 4**).

4. Discussion

At present, more and more biomarkers were analyzed in clinical setting. EGFR, which participates in signaling pathways, has shown to be associated with treatment response [19] [20]. These tumor biomarkers, especially on

Table 3. Multivariate analysis.

	TROP2		TM9SF2		TSPAN6		NGFR	
	HR	p	HR	p	HR	p	HR	p
Gender								
Male	NS		1	0.64	1	0.56	NS	
Female			0.77 (0.25 - 2.31)		1.32 (0.51 - 3.41)			
Age								
<65	NS		1	0.15	1	0.01	NS	
≥65			2.32 (0.72 - 7.43)		0.19 (0.05 - 0.71)			
Histological grade								
WD			1		1			
MD	NS		1.62 (0.50 - 5.29)	0.65	0.86 (0.32 - 2.29)	0.96	NS	
PD			0.84 (0.07 - 9.56)		NA			
Stage								
Early	NS		1	0.01	1	<0.01	NS	
Late			3.88 (1.39 - 10.78)		23.45 (3.48 - 158.00)			
CEA								
<5	NS		1	0.38	1	0.02	NS	
≥5			1.83 (0.46 - 7.18)		4.25 (1.26 - 14.24)			
Score								
Low	NS		1	0.72	1	0.02	NS	
High			1.22 (0.39 - 3.79)		3.75 (1.18 - 11.94)			

NS: not significant. NA: not available.

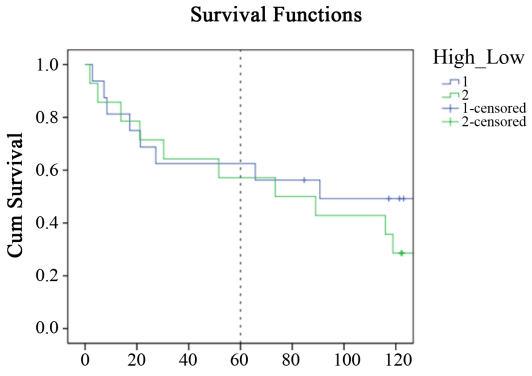
tissue level, were expected to improve earlier diagnosis rate and to make management more individualized [5] [7] [15].

Our study verified four membrane proteins from a secretome dataset in tissue level. The IHC scores of four candidate proteins were markedly different between tumor and adjuvant normal. Although further analysis did not show statistical difference, all of these proteins showed some trends with poorer survival. Except TSPAN6, other three proteins (TROP2, TM9SF2, NGFR) showed some trends with tumor aggressiveness (tumor stage, lymph node metastasis). Statistical insignificance might be due to small sample sizes.

In the literature, several tissue biomarkers had been verified in CRC (**Supplement Table 1**). Most of them are associated with survival or prognosis (**Supplement Table 1**). At present, tissue markers, not serum markers, can be used as a predictor of treatment response. For example, tissue expression of EGFR, which had been verified as biomarker of treatment response [27], was also a biomarker of survival. Survivin, an inhibitor of apoptosis, is known to be expressed in most tumor cell types. Several studies had shown its potential as a target for cancer therapy [42] [43]. Tissue biomarkers, which were tested more widely, seem to have the potential to be integrated in CRC management. Unlike serum markers, tissue markers research is more likely to be a straightforward strategy for treatment markers discovery.

Our data didn't usually show consistency between four candidates and different analyses. And p values were not significant because of small sample sizes. However, marked IHC scoring differences existed between tumor

5-year survival, OS, TROP2

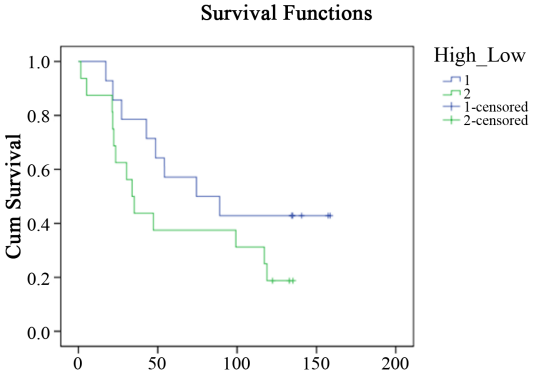


	n	5-year survival (%)	10-year survival (%)
Low expression	16	62.5	50.0
High expression	14	57.1	28.5

P=0.43 (Log Rank test)

(a)

5-year survival, OS, TM9SF2

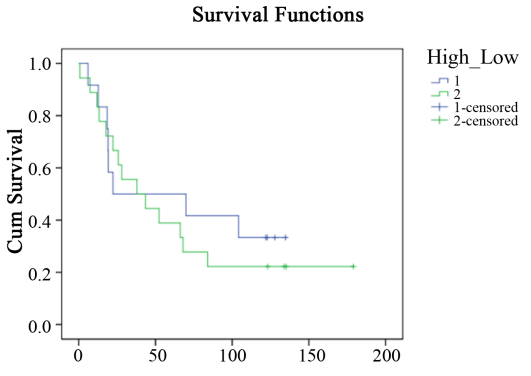


	n	5-year survival (%)	10-year survival (%)
Low expression	14	8/14	6/14
High expression	16	6/16	3/16

P=0. 14 (Log Rank test)

(b)

5-year survival, OS, TSPAN6

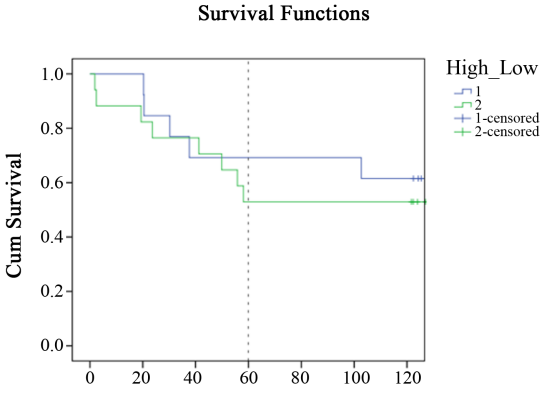


	n	5-year survival (%)	10-year survival (%)
Low expression	12	6/12	4/12
High expression	18	7/18	4/18

P=0.60 (Log Rank test)

(c)

5-year survival, OS, NGFR



	n	5-year survival (%)	10-year survival (%)
Low expression	13	69.2	61.5
High expression	17	52.9	52.9

P=0.62 (Log Rank test)

(d)

Figure 2. Kaplan-Meier survival analysis of different protein expression groups. The 10-year survival rate of high expression and low expression groups were 28.5% vs. 50.0% (p = 0.43) for TROP2 (a), 42.0 % vs. 18.0% (p = 0.14) for TM9SF2 (b), 33.0% vs. 22.0% (p = 0.60) for TSPAN6 (c), and 61.5% vs. 52.9% (p = 0.62) for NGFR (d).

and adjuvant normal, and most candidates showed some trends with tumor aggressiveness and survival. In comparison analysis with CEA, our candidate proteins also showed the potential of improving false negative

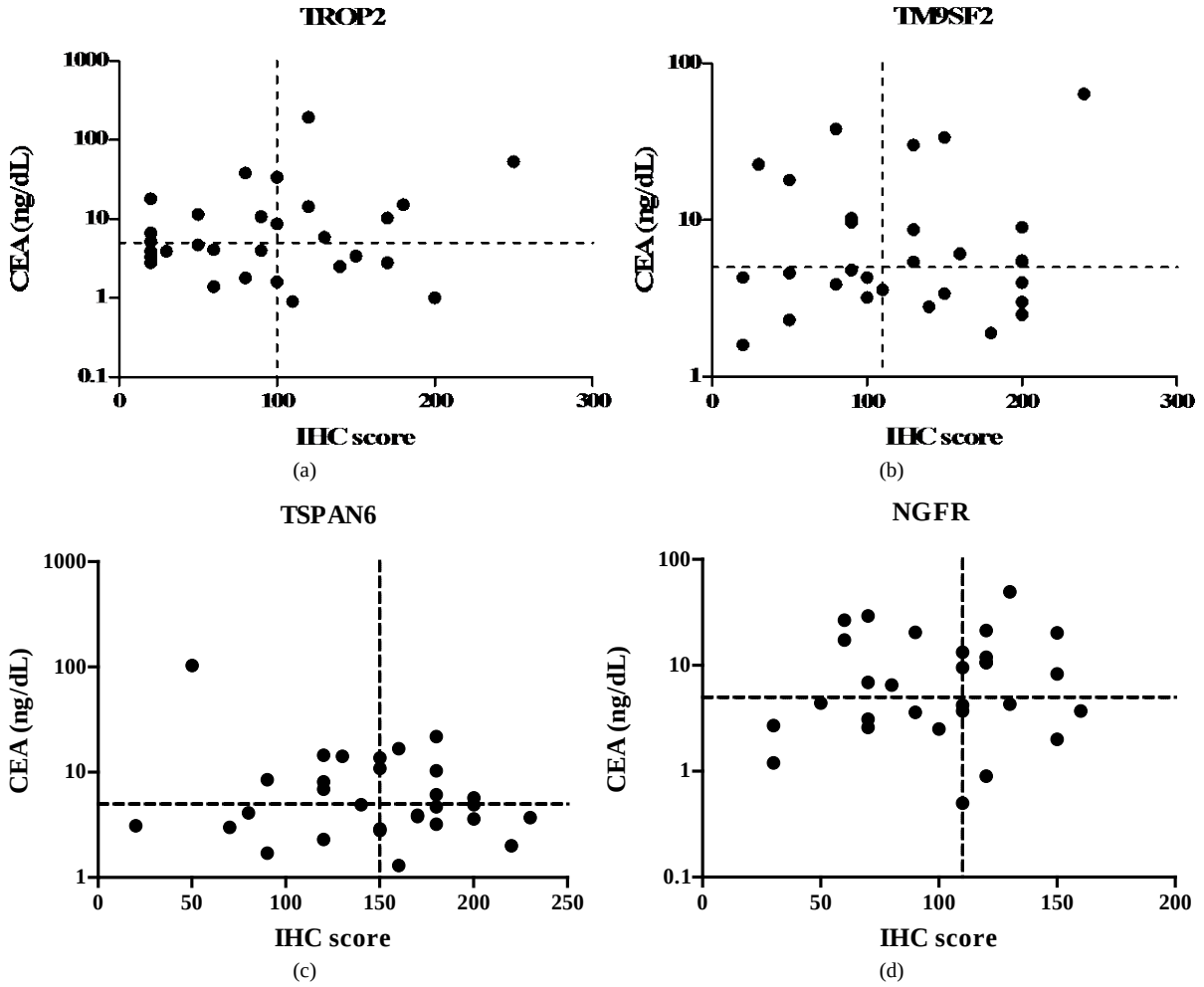


Figure 3. Medium IHC scores were used as cut off values for each proteins. (a) For TROP2, 6 among 16 normal CEA cases had higher TROP2 tissue expression. (b) For TM9SF2, 6 among 15 normal CEA cases had higher tissue expression. (c) For TSPAN6, 11 among 17 normal CEA cases had higher tissue expression. (d) For NGFR, 7 among 14 normal CEA cases had higher tissue expression.

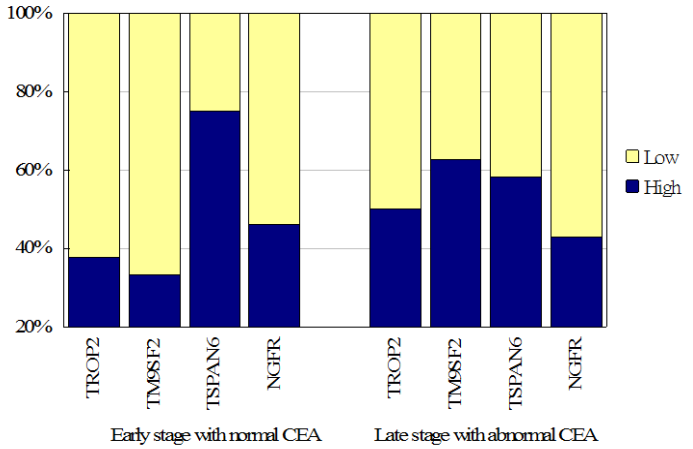


Figure 4. Comparison of the percentages of high and low protein expression. For TROP2 and TM9SF2, more percentages of high protein expression were found in late stage CRC patients with abnormal CEA. For TSPAN6 and NGFR, the associations were reverse.

rate of CEA. More cases to be tested are needful. These four membrane proteins still have the potential to be novel CRC biomarkers. More studies are needed to integrate these proteins in clinical usage. The association between serum and tissue expression is the next interesting issue.

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Supplemental

Table 1. Prioritization and selection of candidate biomarkers from CRC cell secretomes.

Category	Criteria*	Protein name	Gene symbol	Criterion 1	Criterion 2	Criterion 3	Criterion 4
A	1 + 2 or 1 + 3	Tumor-associated calcium signal transducer 2/trophoblast cell surface antigen 2	TACSTD2/ TROP2	Yes	No	[1]-[6]	
		Transmembrane 9 superfamily member 2	TM9SF2	Yes	Yes		
B	1 + 4 or 2 + 3 or 2 + 4	Tetraspanin-6	TSPAN6	No	Yes		[7] [8]
		Bone marrow stromal antigen 2	BST2	No	Yes	[9]-[12]	
		Tumor necrosis factor receptor superfamily member 16	NGFR	No	Yes	[13]-[17]	
		Glia-activating factor	FGF9	No	Yes	[18]-[22]	
		Isoform 3 of Canalicular multispecific organic anion transporter 2	ABCC3**	Yes	No	[23]-[27]	
		Cell surface A33 antigen	GPA33	No	Yes	[28]-[32]	
		55 kDa erythrocyte membrane protein	MPP1	No	Yes		[33]-[36]
		142 kDa protein	PCDH24	No	Yes	[37]	
		Isoform 2 of Ankyrin repeat-rich membrane spanning protein	KIDINS220	No	Yes	[38]	
		Flotillin 2	FLOT2	No	Yes	[39]-[41]	
		Trefoil factor 3	TFF3	No	Yes	[42]-[46]	
		Isoform long of Antigen KI-67	MKI67	No	Yes	[47]-[49]	
		22 kDa protein	TIMP2	No	Yes	[50]-[53]	
		Claudin-3	CLDN3	No	Yes	[54]-[59]	
		Metallothionein-3	MT3	No	Yes	[60]-[62]	
C	2 or 3 or 4	EPHB6 protein	EPHB6	No	No	[63]-[67]	
		Rho-related BTB domain-containing protein 3	RHOBTB3	No	Yes		
		Galactoside 3 (4)-L-fucosyltransferase	FUT3	No	No	[68]-[70]	
		Protein VAC14 homolog	VAC14	No	Yes		
		Isoform 1 of TRAF2 and NCK-interacting protein kinase	TNIK	No	Yes		
		Isoform 3 of Misshapen-like kinase 1	MINK1	No	No	[71]-[73]	
		Protein-tyrosine kinase fyn isoform c	FYN	No	Yes		
		Isoform 2 of protein phosphatase slingshot homolog 3	SSH3	No	Yes		
		Xylosyltransferase 1	XYLT1***	Yes	Yes		
		70 kDa protein	SLC6A6	No	Yes		
		Protein APCDD1	APCDD1	No	Yes		
		Isoform CSBP1 of Mitogen-activated protein kinase 14	MAPK14	No	Yes		
		Ephrin type-B receptor 3	EPHB3	No	Yes		
		Ataxia telangiectasia mutated protein isoform 2	ATM	No	Yes		
		Stromal cell-derived factor 2	SDF2	No	No	[74] [75]	
		Perforin-1	PRF1	No	No	[76]	
		Inhibin beta B chain	INHBB	No	No	[77]-[79]	

Continued

		Coagulation factor XIII A chain	F13A1	No	No	[80]-[82]
		Low-density lipoprotein receptor-related protein 4	LRP4	No	Yes	
		CDC42 binding protein kinase alpha	CDC42BPA	No	No	[83]
		Isoform long of Glycylpeptide N-tetradecanoyltransferase 1	NMT1	No	Yes	
		Cell growth regulator with EF hand domain protein 1	CGREF1	No	Yes	
		Isoform 1 of protein KIAA1199	KIAA1199	No	No	[84]-[85]
		AP1B1 protein	AP1B1	No	No	[86]-[89]
		Ladinin 1	LAD1	No	Yes	
		Isoform 1 of von Willebrand factor A domain-containing protein 2	VWA2	No	No	[90]
		Isoform 1 of Transmembrane protein 16A	TMEM16A	No	No	[91] [92]
		Isoform 2 of Condensin-2 complex subunit G2	NCAPG2	No	Yes	
		Prostatic acid phosphatase	ACPP	No	Yes	
		9 kDa protein	RPS27	No	No	[93]-[96]
		Isoform 2 of General transcription factor 3C polypeptide 5	GTF3C5	No	No	[97] [98]
		Isoform 2 of Transmembrane and TPR repeat-containing protein 3	TMTC3	No	Yes	
		Catenin, beta like 1	CTNNBL1	No	Yes	
C	2 or 3 or 4	Ras GTPase-activating-like protein IQGAP3	IQGAP3	No	No	[99] [100]
		Isoform 1 of PDZ domain-containing protein 11	PDZD11	No	Yes	
		Lipocalin 2	LCN2	No	Yes	
		cDNA FLJ46245 fis, clone TESTI4020596, highly similar to Homo sapiens calpain 5	CAPN5	No	No	[101] [102]
		Proliferating-cell nucleolar antigen p120	NOL1	No	No	[103]-[105]
		Isoform 1 of Pregnancy-specific beta-1-glycoprotein 11	PSG11	No	No	[106] [108]
		Isoform 1 of Choline transporter-like protein 1	SLC44A1	No	No	[109]
		Phosphoglycerate mutase 2	PGAM2	No	No	[110] [111]
		Isoform A of Nucleoporin SEH1-like	SEH1L	No	Yes	
		Fibroblast growth factor 19	FGF19	No	No	[112] [113]
		Isoform 2 of Chloride intracellular channel protein 5	CLIC5	No	No	[114]
		36 kDa protein	MFNG	No	No	[115]
		Eukaryotic translation initiation factor 1A, Y-chromosomal	EIF1AY	No	Yes	
		Calcium and integrin-binding protein 1	CIB1	No	Yes	
		Neutral amino acid transporter A	SLC1A4	No	Yes	
		Receptor-type tyrosine-protein phosphatase epsilon precursor	PTPRE	No	Yes	

*Criteria: 1) Proteins detected in the high-confidence human plasma proteome reference set established in 2011 [States *et al*., 2006] (http://www.hupo.org). 2) Proteins overexpressed in CRC tissue specimens in the Human Protein Atlas (HPA) dataset [Bjorling *et al*., 2008] (http://www.proteinatlas.org). 3) Proteins up-regulated in CRC in published references. 4) Functions as secreted proteins, or involving in apoptosis/signal transduction. **ABCC3 was set in category B due to previous literature lacking positive association in CRC. ***XYLT1 was set in category C because it is an enzyme which was not a favorable candidate for cancer biomarker.

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