研究報告: 利用血清小分子核糖核酸對二型糖尿病患者中肝癌的早期檢測

Early detection of liver cancer in type 2 diabetes using serum microRNA

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簡介

糖尿病和癌症都有複雜的病因並可能互相影響。二型糖尿病患者患癌的風險較沒有糖尿病的患者高 1.3 至 3 倍 [1－3]。糖尿病人患癌致死不容忽視，本地約有四分之一糖尿病死亡個案來自癌症 [4，5]。香港中文大學內科及藥物治療學系的流行病學研究發現二型糖尿病患者的高血糖可能是患癌風險上升的主因 [6－8]。我們在 1995 年建立了香港糖尿病登記這個定期更新的資料庫，紀錄糖尿病患者的臨床檢驗數據和病情變化 [4，7]，用以提升治療糖尿病的效果。我們利用這個資料庫的數據，查證各種與癌症相關的風險因素，發掘出二型糖尿病患者患上癌症前的一些共同特徵。首先，血液中的脂肪水平和白血球數量可以預測糖尿病患者患上癌症的風險，並呈現非線性（non-linear）相關 [9]。二型糖尿病患者患上癌症的風險隨血液中總膽固醇上升而下降，直至血液中總膽固醇高於 4. 3 mmol／L 時，患上癌症的風險不再下降，趨於平穩。血液中白血球數量與患癌的風險呈現 V 型關係，血液中白血球數量低於 5. $8x10^9$／L 時患癌風險顯著增加 [9]。我們綜合了各種患癌的風險因素，包括年齡、吸煙狀況和血液中的脂肪水平和白血球數量，開發出二型糖尿病患者患癌的風險方程式，經過在二型糖尿病患者中驗證，在接收者操作特徵曲線（receiver’s operating characteristic curve）下的面積（aROC）達到 0.71 [10]。再者，我們還發現低密度膽固醇（LDL－C）和患癌風險有非線性關係 [11]，而且在服用腎素－血管收縮素系統（RAS，renin-angiotensin system）抑制劑和他汀類（statin）藥物的病例中大幅少 [12]。這個發現和我們在大鼠身上切除單腎（UNX，uninerphrectomixed）的實驗結果不謀而合 [13]。大鼠切除單腎後，出現糖尿、血脂異常、腎衰竭和腎臟腫瘤等徵狀，服用 RAS 抑制劑後，這些徵狀大幅緩解，腫瘤數量減少，膽固醇合成和胰島素生長因子（IGF－1）的活性恢復正常，暗示 RAS 系統、膽固醇合成的調控和胰島素生長因子訊號傳遞之間有更深入的相互作用，影響癌症在二型糖尿病患者的發展 [14]。我們從隨訪跟進的二型糖尿病病例中發現，糖化血紅素（HbA1c）每增加百分之一，患上癌症的風險相應增加百分之十八 [7]。從香港糖尿病登記中的病例顯示，約有一半的二型糖尿病癌症病例是消化系統的癌症，包括肝癌和大腸直腸癌。我們曾經進行研究，發現有慢性乙型肝炎的二型糖尿病患者患上肝癌的風險大幅增加，風險比率在血糖控制不善（HBA1c ≥ 7%）的患者中由 3.74 增至 74.96 [15]。

小分子核糖核酸（microRNA）是一類由 19 至 28 個核苷酸組成可以調控基因表達的非編碼核糖核酸。大概而言，microRNA 通過影響轉錄後的機制來抑制基因表達。microRNA 通過和信使核糖核酸（mRNA）3’末端非編碼區（3’UTR）上的標靶序列結合，引發信使核糖核酸降解或抑制合成蛋白質的轉譯作用 [16]。同一種 microRNA 可以作用於多個不同基因的信使核糖核酸，因而調控多個基因和生物途徑，包括細胞增長 [17]、凋亡作用 [18] 和癌細胞代謝 [19，20]，起關鍵作用。不少研究都發現 microRNA 在癌症和一些疾病中表達異常 [21－23]。例如 miR-221/222 在一些腫瘤中水平升高。在前列腺癌中，miR-221/222 針對一個抑制細胞周期的調控基因 p27Kip1 起作用，降低其表達水平，從而促進癌細胞增長。當 miR-221/222 受到抑制後，癌細胞增長亦相應受制 [17]。在乳癌 [24] 和肝癌 [25] 細胞中也發現類似

的調控機制。另一方面，常見 miR-16 在一些癌細胞中受到抑制。在白血病細胞中，miR-16 可以抑制有抗凋亡作用的基因 Bcl-2，低水平的 miR-16 令癌細胞不受凋亡作用影響，持續生長 [18]。microRNA 不單在細胞內起作門，癌細胞還可以分泌 microRNA 進入血液中，形成小囊泡可作用於遠端的細胞和器官。

近年的研究顯示檢測血清中 microRNA 可以成為癌症的生物指標，具有臨床應用價值。找尋癌症的生物指標用於預報和早期發現癌症是生物醫學研究中一個快速發展的領域 [26－28]。microRNA 是近年這方面研究的焦點，因為癌細胞中 microRNA 在各種調控機制的上游起關鍵作用，是成癌機理的一部份 [16]。再者，癌細胞常見的染色體缺失區域往往包括聚集一起的 microRNA 基因 [29－31]。microRNA 表達調控失常在癌細胞中頗為常見 [21，32]，microRNA 與癌細胞的密切關係令其成為早期發現癌症的研究的主要目標。在 microRNA 與癌症的研究中，已經在癌細胞的分型特徵 [33]、偵測和預後 [23，34] 方面花了不少功夫。最近有研究報告利用實時 PCR 技術在血清中檢測 microRNA，發現和肺癌和大腸直腸癌相關的 microRNA 表達特徵 [35]。同一個報告還顯示血清中的 microRNA 表達水平穩定可靠，結合實時 PCR 技術有望發展成一個簡單、識別性高的非侵入性癌症檢測技術。

目標

我們的目標是開發一組可以早期預測二型糖尿病患者患上肝癌的小分子 RNA（microRNA）特徵，並為這組高危患者開發肝癌的早期檢測分析。

方法

我們從香港糖尿病登記中選出潛在的肝癌病例的血清樣本提取 microRNA 用於微陣列研究，以發現用於早期檢測二型糖尿病肝癌患者的 microRNA 特徵。對於第一步微陣列研究，選擇了 10 個潛在的二型糖尿病患上肝癌的病例，對照是 10 個沒有癌症的二型糖尿病病例。兩組在年齡、性別、病齡、身高體重指數（BMI）上匹配，患癌病例的血清樣本在癌症確診之前 1 至 5 年收集。從所選病例的血清樣本中提取 microRNA 後，使用 Affymetrix GeneChip miRNA 4.0 微陣列檢測 microRNA 在血清中的水平，利用 GeneSpring 軟件進行數據分析，選出 5 至 10 個 microRNA，使用實時 PCR（real-time PCR）在 400 個 T2D 病例中進一步驗證核實。

结果

1. 利用微陣列找出二型糖尿病肝癌患者的 microRNA 特徵

我們從香港糖尿病登記中選出潛在的肝癌病例的血清樣本提取 microRNA 用於微陣列研究，以期找出二型糖尿病患者患上肝癌的 microRNA 早期檢測特徵。表

一是所選病例和對照組的比較。利用 10 個肝癌病發前 1 至 5 年收集的二型糖尿病血清樣本，配合 10 個在年齡、性別、BMI 和病況相若的血清樣本作對照，我們用 Trizol 試劑從血清中提取核糖核酸（RNA）作檢測比較。整個過程遵照標準操作步驟，並加入外源的 ath-miR-172a（microRNA）用作質量監控。我們採用了 Affymetrix 公司 Gene Chip miRNA 4.0 微陣列去檢測血清 microRNA 水平，委託香港中文大學李嘉誠健康科學研究所中心實驗室按照標準的步驟進行標記、雜交、清洗和掃描，測定血清中 microRNA 水平。得到的數據用 Gene Spring v.13 軟件進行分析，以期找出二型糖尿病肝癌患者的 microRNA 早期特徵。

經過分析，Affymetrix Gene Chip miRNA 4.0 微陣列檢測到 4411 種 microRNA 和非編碼 RNA。其中 271 個 microRNA 和 microRNA 前體（precursor）統計上在患癌組和對照組之間有顯著差異。當中 17 個 microRNA 在患癌組明顯增加了百分之五十或以上，列於表二。

2. 利用實時 PCR 驗證核實 microRNA 表達水平

我們從香港糖尿病登記中選出更多的病例去進一步驗證核實從微陣列研究發現的結果。用於驗證的病例包括 109 個二型糖尿病患上肝癌的病例，以及 236 個和肝癌病例在年齡、性別、BMI、HbA1c 水平和病況相若的非癌症病例。此外還包括 139 個潛在大腸癌病例，以測試在肝癌中找到的 microRNA 特徵是否在其它癌症中出現，表三比較了三組病例的基本參數。我們從收集的血清樣本中提取 RNA，然後用實時 PCR 定量 microRNA 的水平。

我們用實時 PCR 技術定量測試了在第一部分微陣列研究中顯示最大增幅的 5 個 microRNA，包括 miR-122-5p、miR-4454、miR-486-5p、miR-92a-3p 和 miR-4532，並在每個樣本中加入相同數量的外源 ath-miR-172a，用以修正誤差。首先用血清中的 RNA 為模板，用 Taqman Advanced miRNA cDNA synthesis kit 試劑盒合成 cDNA，然後用 Taqman Advanced miRNA assays 嚴格按照生產商規定的步驟進行實時 PCR 定量測試。其中只能在少於百分之十的樣本中檢測到 miR-4454，因此這一 microRNA 不再跟進。其餘 microRNA 都能在大部分樣本中檢測到。個別 microRNA 在個別樣本中水平太低未能檢測到，數值上按等於零處理。實時 PCR 定量測試的結果用 SPSS v.22 軟件進行統計分析。

我們用方差分析（ANOVA）比較三組病例的血清 microRNA 水平，顯示 miR-122-5p、miR-92a-3p 和 miR-4532 平均水平都有明顯差異。二型糖尿病肝癌患者血清中的 miR-122-5p 平均水平比沒有患癌的二型糖尿病患者高 2 倍（P = 0.0002，圖一）。相較之下，二型糖尿病肝癌患者血清中的 miR-92a-3p 水平只升高了 48%（P = 0.028，圖一）而 miR-4532 在二型糖尿病肝癌患者血清中升高了 1.9 倍（P = 0.0297，圖一）。在二型糖尿病大腸癌患者中，幾個 microRNA 的血清水平都沒有明顯差異。當二型糖尿病肝癌患者和沒有肝癌的二型糖尿病患者（沒有患癌的二型糖尿病患者加上二型糖尿病大腸癌患者）比較，四種 microRNA 水平都有顯著增加（表四）。

如果二型糖尿病肝癌患者加上大腸癌患者加在一起和沒有患癌的二型糖尿病患者作比較，miR-122-5p 和 miR-4532 的血清水平都明顯增加。

結論

四個經測試的 microRNA（miR-122-5p、miR-486-5p、miR-92a-3p 和 miR-4532）在潛在二型糖尿病肝癌患者中的血清水平都有增加，其中以 miR-122-5p 最明顯突出。miR-122-5p 很有潛力成為預測二型糖尿病患者患上肝癌的生物標記。我們需要進一步深入研究，證實 miR-122-5p 的重要性和在臨床上的應用，並以此開發早期發現二型糖尿病患者患上肝癌測試。

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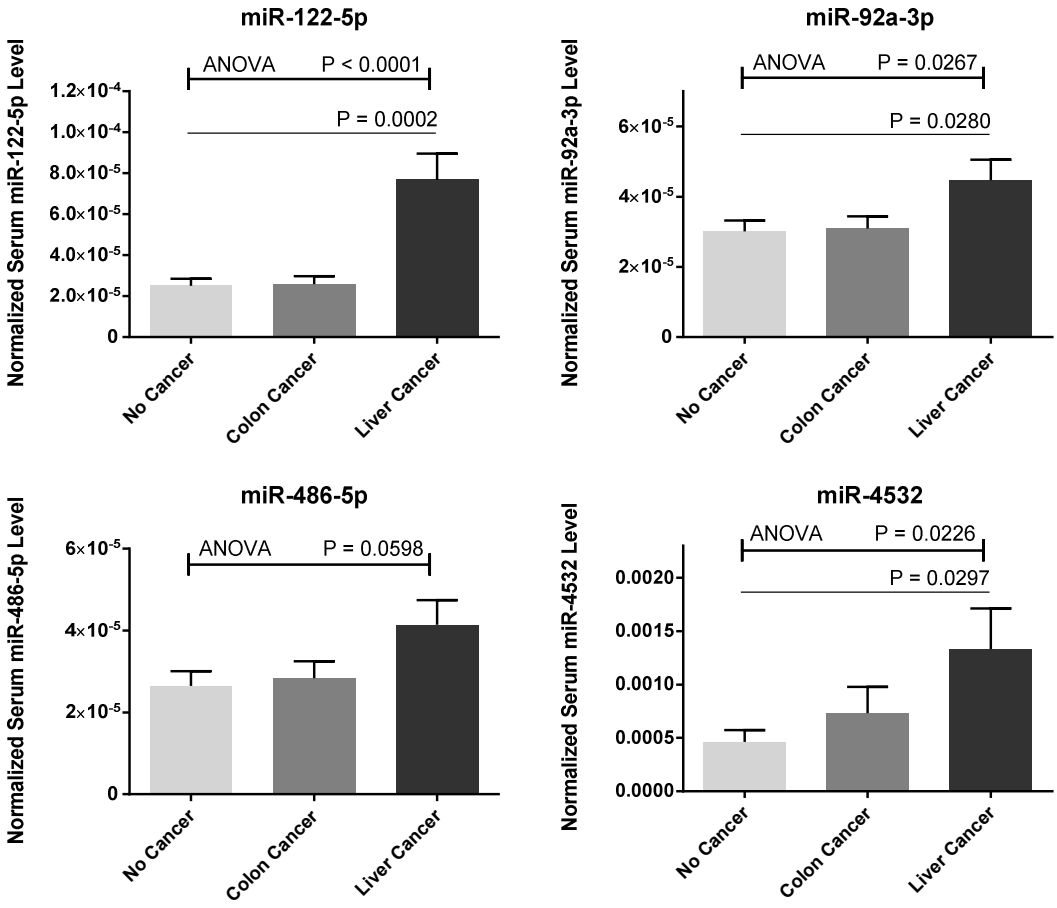

圖一　二型糖尿病患者的血清 microRNA 水平。圖示經標準化的血清 miR-122-5p, miR-92a-3p, miR-486-5p 和 miR-4532 在二型糖尿病無癌症組、二型糖尿病肝癌組和二型糖尿病大腸癌組的相對水平，以 Mean ± SEM 表示。

表一 進行微陣列研究的病例比較

	T2D No cancer 糖尿病無癌症	T2D Liver cancer 糖尿病肝癌	*P*
數目 (N)	10	10	
性別 (F:M)	4 : 6	4 : 6	
年齡 (歲)	62.40 ± 9.81	64.30 ± 7.96	0.640
病齡 (年)	7.90 ± 5.20	7.00 ± 5.27	0.705
BMI	24.48 ± 2.95	25.02 ± 3.11	0.697
HbA1c (%)	7.83 ± 1.22	8.01 ± 2.91	0.860
總膽固醇 (mmol/L)	5.99 ± 0.82	5.15 ± 1.76	0.214
白血球計數 (10^9 cells/L)	6.58 ± 1.72	7.62 ± 4.88	0.534
癌前期 (年)		3.07 ± 1.41	

以 Mean ± SD 表示

表二 二型糖尿病潛在肝癌患者血清 microRNA 變化綜覽

Micro RNA	*P*	調控方向	變化水平（倍）
miR-122-5p	4.46e-05	上升	3.14
miR-4454	0.0157	上升	2.10
miR-486-5p	0.0148	上升	2.10
miR-92a-3p	0.0166	上升	2.09
miR-4532	0.0124	上升	2.09
miR-320b	0.0020	上升	1.96
miR-7110-5p	0.0368	上升	1.85
miR-320c	0.0075	上升	1.78
miR-185-5p	0.0156	上升	1.76
miR-320a	0.0103	上升	1.75
miR-4674	0.0225	上升	1.73
miR-3663-3p	0.0161	上升	1.69
miR-93-5p	0.0047	上升	1.60
miR-4492	0.0275	上升	1.57
miR-320d	0.0228	上升	1.56
miR-22-3p	0.0105	上升	1.55
miR-17-5p	0.0073	上升	1.50

表三 進行 qPCR 驗證的病例比較

	T2D No cancer 糖尿病無癌症	T2D Colon cancer 糖尿病大腸癌	T2D Liver cancer 糖尿病肝癌	ANOVA *P*[1]	*P*[2]
數目 (N)	236	139	109		
性別 (F:M)	65 : 171	63 : 76	21 : 88		
年齡 (歲)	60.15 ± 11.23	64.68 ± 8.72	58.92 ± 10.37	0.000	0.333
病齡 (年)	6.41 ± 6.04	7.37 ± 6.44	6.31 ± 5.56	0.264	0.889
BMI	24.46 ± 3.61	25.47 ± 3.60	23.74 ± 2.85	0.000	0.068
HbA1c (%)	7.92 ± 1.85	7.81 ± 1.744	7.91 ± 1.87	0.838	0.971
總膽固醇 (mmol/L)	5.25 ± 1.01	5.16 ± 1.13	4.90 ± 1.20	0.031	0.008
白血球計數 (10^9 cells/L)	7.37 ± 1.94	7.23 ± 1.91	6.68 ± 2.65	0.030	0.025
癌前期 (年)		5.92 ± 4.72	5.17 ± 4.53	0.210	

以 Mean ± SD 表示

[1] 用 ANOVA 進行三組比較

[2] 用 t-test 比較糖尿病無癌組和糖尿病肝癌組

表四 各組 microRNA 血清水平比較

	Serum level in T2D no cancer 糖尿病無癌症	Serum level in T2D colon cancer 糖尿病大腸癌	Serum level in T2D liver cancer 糖尿病肝癌	三組 ANOVA *P*	糖尿病肝癌對糖尿病無癌症 *P*	糖尿病肝癌對糖尿病大腸癌 *P*	糖尿病肝癌對糖尿病無肝癌 *P*	糖尿病有癌症對糖尿病無癌症 *P*
miR-122-5p	2.50e-05 ± 3.54e-06	2.58e-05 ± 3.84e-06	7.67e-05 ± 1.30e-05	< 0.0001	0.0002	0.0434	0.0002	0.0014
miR-92a-3p	3.01e-05 ± 3.04e-06	3.10e-05 ± 3.41e-06	4.47e-05 ± 5.83e-06	0.0267	0.0280	0.0002	0.0243	0.1223
miR-486-5p	2.65e-05 ± 3.60e-06	2.83e-05 ± 4.16e-06	4.14e-05 ± 6.03e-06	0.0598	0.0347	0.0761	0.0330	0.1325
miR-4532	4.60e-04 ± 1.13e-04	7.32e-04 ± 2.46e-04	1.33e-03 ± 3.80e-04	0.0226	0.0297	0.1871	0.0546	0.0291

血清 microRNA 水平為標準化後相對數值，以 Mean ±SEM 表示

附錄: 財務報告

開支明細表

日期	公司	發票號碼	採購物件／服務	金融 (HK$)
1/4/2016	Life technologies	16124346	primers for miRNA qPCR	2,121.6
18/5/2016	LKS core laboratory		Affymetrix miRNA 4.0 Microarray service	70,000.00
24/5/2016	Life technologies	16128067	Taqman advance miRNA cDNA synthesis kit	3,607.40
14/6/2016	Life technologies	16129514	Glycogen	1,420.00
14/6/2016	Life technologies	16129529	Taqman fast advance master mix	4,213.45
15/6/2016	Bio-Gene Tech Ltd	IN1605878	384 well plates for PCR	1,260.00
12/8/2016	Tech Dragon Ltd	TIV201608179	Oligo primers	873.60
17/8/2016	Bio-Gene Tech Ltd	IN1608156	96-well and 384-well PCR plates	2,120.00
17/8/2016	Bio-Gene Tech Ltd	IN1608197	MirX miRNA first strand synthesis kit	4,190.00
23/8/2016	Tech Dragon Ltd	TIV201608320	Oligo primers	886.60
29/8/2016	Life technologies	16134928	Taqman advance miRNA cDNA synthesis kit	14,429.60
7/9/2016	Bio-Station Ltd	BS1609010	SYBR Premix Ex Taq master mix	1,980.00
15/9/2016	Life technologies	16136205	Taqman fast advance master mix, 10 ml	7,586.25
16/9/2016	Life technologies	16136246	Taqman miRNA assay	4,243.20
20/9/2016	Life technologies	16136520	Taqman advance miRNA cDNA synthesis kit	14,429.60
20/9/2016	Tech Dragon Ltd	TIV201609240	RNA oligo	1,672.00
23/9/2016	Life technologies	16136774	Taqman Advance miRNA assays	12,729.60
7/10/2016	Life technologies	16137654	Taqman Advance miRNA assays	8,486.40
11/10/2016	Tech Dragon Ltd	TIV201610071	RNA oligo	1,558.00
25/10/2016	Bio-Gene Tech Ltd	IN1610538	P10 pipette tips	720.00
26/10/2016	Tin-Hang Tech. Ltd.	IN16-018573	SP000125, 10 mg	1,060.00
11/11/2016	Eppendorf	95032743	Multi channel pipette	6,222.40
24/11/2016	Eppendorf	95032930	Pipette tips and consumables	2,033.10
17/11/2016	Life technologies	16140711	Mitosox Red stain	2,548.00
11/1/2017	Bio-Gene Tech Ltd	IN1700429	96-well plate and sealing films	1,760.00
23/2/2017	Life technologies	17147200	Trizol and RNase free water (partial)	49.20
			總數	172,200.00

Completion Report for Research Grant supported by HKACS (The Hong Kong Anti-Cancer Society)

Project title:

Early detection of liver cancer in type 2 diabetes using serum microRNA

Investigators:

Alice P.S. Kong (Principal investigator)

Juliana C.N. Chan (Co-investigator)

Ronald C.W. Ma (Co-investigator)

Andrea O.Y. Luk (Co-investigator)

Heung Man Lee (Co-investigator)

Milestones

Study starting date: 1 Dec 2015

Study completion date: 30 Nov 2016

Grant amount

HK$ 198,030

Introduction

Diabetes and cancer are complex diseases which share common biological pathways. Patients with type 2 diabetes (T2D) have 1.3 to 3 fold increase in cancer risk compared to non-diabetic counterparts [1-3]. Cancer is now the leading cause of death in diabetes and accounts for 25% of deaths in our local diabetic population [4, 5]. Epidemiological studies support a possible link between hyperglycemia and cancer risk for all-site cancer in T2D patients [6-8]. Since 1995, we established a prospective cohort, the Hong Kong Diabetes Registry [4, 7], as part of a quality improvement program and for the study of the clinical course, phenotype and clinical outcomes for Chinese patients with T2D. Using the data from this well-characterized cohort, we examined risk factors of cancer in T2D to elucidate abnormalities which may precede onset of cancer. First, we reported that lipids and white blood cell (WBC) count were predictors of cancer and their risk associations with cancer exhibit non-linear patterns [9]. Increasing total cholesterol correlated with decreased cancer risk at total cholesterol < 4.3 mmol/L and beyond that point, the cancer risk is relatively steady. WBC count was associated with cancer in a V-shaped manner with marked increase in cancer risk at WBC count <5.8×10^9 cells/L [9]. Using total cholesterol level, WBC count, age and smoking status, our group has developed and validated a cancer risk score for type 2 diabetic patients, which achieved an area under the receiver's operating characteristic curve (aROC) of up to 0.71 [10]. Additionally, we found non-linear risk association of cancer with low-density lipoprotein cholesterol (LDL-C) [11] which was greatly attenuated by inhibition of the renin-angiotensin system (RAS) and statin therapy [12]. This result was corroborated in our animal study of uninerphrectomized (UNX) rats [13] which showed a combined phenotype of hyperglycemia, dyslipidemia, renal impairment and high incidence of renal cancer. Treatment of the UNX rats with inhibitors of RAS decreased cancer formation and restored the activated cholesterol synthesis and IGF-I pathway, suggesting possible interactions of RAS, cholesterol homeostasis and IGF-I in cancer development in T2D [14]. In prospective analysis, we found that each 1% increase in HbA1c was associated with 18% increased hazard ratio in cancer risk [7]. Our data from the Hong Kong Diabetes Registry revealed that about half of the T2D cancer cases are cancers from the gastrointestinal system including liver and colorectal cancers. In a study for the risk of T2D patients with chronic hepatitis B infection, we showed that the hazard ratio for the patients to develop hepatocellular carcinoma dramatically increased from 3.74 to 74.96 with suboptimal glycemic control (HbA1c $\geq$ 7 %) [15].

MicroRNA (microRNA) is a family of small noncoding RNA 19 to 28 nucleotides in length that can regulate gene expression. Generally, miRNA suppress gene expression through post-transcriptional mechanisms. miRNA binds to the target sequence at the 3' untranslated region (3'UTR) of mRNA to trigger either RNA cleavage or translational inhibition [16]. One miRNA can have multiple targets in different regulatory pathways in cell proliferation [17], gene expression, apoptosis [18] and cancer development [19, 20]. Altered expression of miRNA has been reported in many cancer and disease conditions [21-23]. High level of miR-221/222 is reported in many tumors. In prostatic cancer, miR-221/222 target the 3'UTR of cell cycle suppressor gene p27Kip1 and down regulate its expression. Inhibition of miR-221/222 leads to growth inhibition in cancer cells [17]. Similar mechanisms are also reported in breast cancer [24] and liver cancer cells [25]. By

contrast, miR-16 is commonly found down-regulated in many cancer cells. In leukemia cells, miR-16 inhibits the expression of the apoptotic suppressor Bcl-2 to promote tumor growth and progression [18]. Not only miRNA can work intracellular to inhibit gene expression, they can also be secreted by cancer cells into the blood as vesicles and possibly mediate its function in remote cells and organs.

Recent studies showed that miRNA in serum can be used as a marker for cancers with potential clinical application. The search of cancer markers for prediction and early diagnosis is a rapidly growing area in biomedical research [26-28]. miRNA is the main focus because miRNA is the key change upstream in the regulatory hierarchy in cancer cells that leads to tumorigenesis [16]. Besides, miRNA clusters are found to associate with chromosomal regions commonly deleted in cancers [29-31]. Dysregulation of miRNA expression has been reported in cancer cells [21, 32]. The close association of miRNA with cancer cells prompts it as a prime target for early cancer detection. There is an enormous effort to develop a miRNA signature of cancer typing [33] and miRNA expression profiles for cancer detection [23, 34]. Direct detection of miRNA in serum by quantitative real-time PCR (qRT-PCR) shows a specific expression patterns that can be associated with lung cancer and colorectal cancer [35]. The same study [35] also shows that serum miRNA levels are stable and reproducible, which means qRT-PCR measurement of serum miRNA level is a simple and specific non-invasive method for cancer detection.

Objective

We aim to develop a panel of microRNA markers predictive of liver cancer in patients with T2D and develop an early detection assay for liver cancer in this group of at-risk patients.

Methodology

We used serum samples from prospective cancer cases selected from Hong Kong Diabetes Registry to extract miRNA for microarray study to discover a signature for early detection of liver cancer in patients with T2D. For the first step microarray study, 10 T2D prospective liver cancer cases were selected. The controls were 10 cancer free cases matched for age, sex and disease duration of diabetes. miRNA were extracted from the serum samples of the selected cases for microarray study. For this first step, serum samples collected 1 to 5 years before the first reported liver cancer event were selected. The Affymetrix GeneChip miRNA 4.0 microarray were used. GeneSpring program was used for data analysis to select 5 to 10 miRNA markers for further validation. The selected miRNA markers were validated in 400 T2D cases using real-time PCR.

Results

1. Use of microarray to identify serum microRNA associated with liver cancer in type 2 diabetes

Serum samples from prospective liver cancer cases were selected from Hong Kong Diabetes Registry to extract miRNA for microarray study to discover a signature for early detection of liver cancer in patients with T2D. Table 1 summarizes the characteristics of the selected cases. 10 Prospective liver cancer T2D cases with the lead time between 1 to 5 years were selected and 10 T2D cancer free cases with matched age, sex, disease duration and BMI were selected as control. Serum microRNA were extracted using the Trizol reagent with standard procedures and ath-miR-172a was spiked-in during the extraction as an internal control. The Affymetrix Gene Chip miRNA 4.0 microarray were used for identification of the serum microRNA marker for liver cancer in T2D cases. All labelling, hybridization and washing procedures were carried out by the staff of the Core Laboratory of the Li Ka Shing Institute of Health Sciences, CUHK according to standard protocols. The data were analysed using the software Gene Spring v.13.

Using the Affymetrix Gene Chip miRNA 4.0 microarray, 4411 microRNA or related non-coding RNA were detected. Among the detected microRNA or microRNA precursors, 271 showed a significant difference between the T2D liver cancer group and the T2D cancer free group. 17 microRNA showed at least 50% increase in the T2D liver cancer group (Table 2).

2. Validation of microRNA level using quantitative real-time PCR

Additional cancer free T2D and T2D liver cancer cases were selected for validation of the results from the microarray study using quantitative real-time PCR. The selected cases are matched with age, disease duration, BMI and HbA1c levels. A total of 109 T2D liver cancer cases and 236 T2D cancer free cases were selected. We also included 139 prospective colon cancer cases from Hong Kong Diabetes Registry to test if T2D colon cancer and liver cancer cases have common serum microRNA markers. The characteristics of the selected cases are listed in Table 3.

The top five microRNA showing the biggest fold change in the microarray study, miR-122-5p, miR-4454, miR-486-5p, miR-92a-3p and miR-4532 were tested using the Taqman real-time qPCR with the spiked-in ath-miR-172a as internal control. Serum microRNA samples were converted to first strand cDNA using the Taqman Advanced miRNA cDNA Synthesis kit and the Taqman Advanced miRNA Assays were used for subsequent real-time qPCR with standard protocols from the manufacturer. Among the tested microRNA, miR-4454 were only detected in less than 10% of the samples and dropped for further analysis. The other microRNA tested were detectable in the majority of the cases. The serum level of the undetected miRNA was regarded as zero during analysis. Statistical analysis of the qPCR results were carried out using SPSS v.22.

When comparing the mean serum level of microRNA levels among different groups using ANOVA test, serum levels of miR-122-5p, miR-92a-3p and miR-4532 showed significant difference. Mean serum level of miR-122-5p in T2D liver cases were 2 times

higher than the level of cancer free T2D cases (P = 0.0002, Figure 1). Serum levels of miR-92a-3p were only 48% higher in the T2D liver cancer cases when comparing to the cancer free T2D cases (P = 0.028, Figure 1) while serum level of miR-4532 were 1.9 times higher in the T2D liver cancer cases (P = 0.0297, Figure 1). Serum levels of the tested microRNA in T2D colon cancer cases showed no difference to the cancer free T2D cases. When the T2D liver cancer cases tested against the T2D cases without liver cancer (T2D colon cancer cases and cancer free T2D cases combined), all 4 microRNA tested showed significant increase in serum levels (Table 4). When the T2D liver cancer and T2D colon cancer cases are combined to test against the cancer free T2D cases, miR-122-5p and miR-4532 showed significant increase.

Conclusion

All four miRNA tested (miR-122-5p, miR-92a-3p, miR-486-5p and miR-4532) showed increased level in the serum of prospective liver cancer T2D cases with miR-122-5p being most significant. miR-122-5p may potentially be a specific biomarker for early detection of liver cancer in T2D. Further studies are required to further verify the role and explore the clinical utility of miR-122-5p in early detection of liver cancer in patients with T2D.

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Figure 1. Normalized serum microRNA levels in T2D liver and colon cancers cases and T2D no cancer cases. The bar chart showed the normalized serum levels of miR-122-5p, miR-92a-3p, miR-486-5p and miR-4532 in T2D liver cancer, T2D colon cancer and T2D no cancer cases (mean ± SEM).

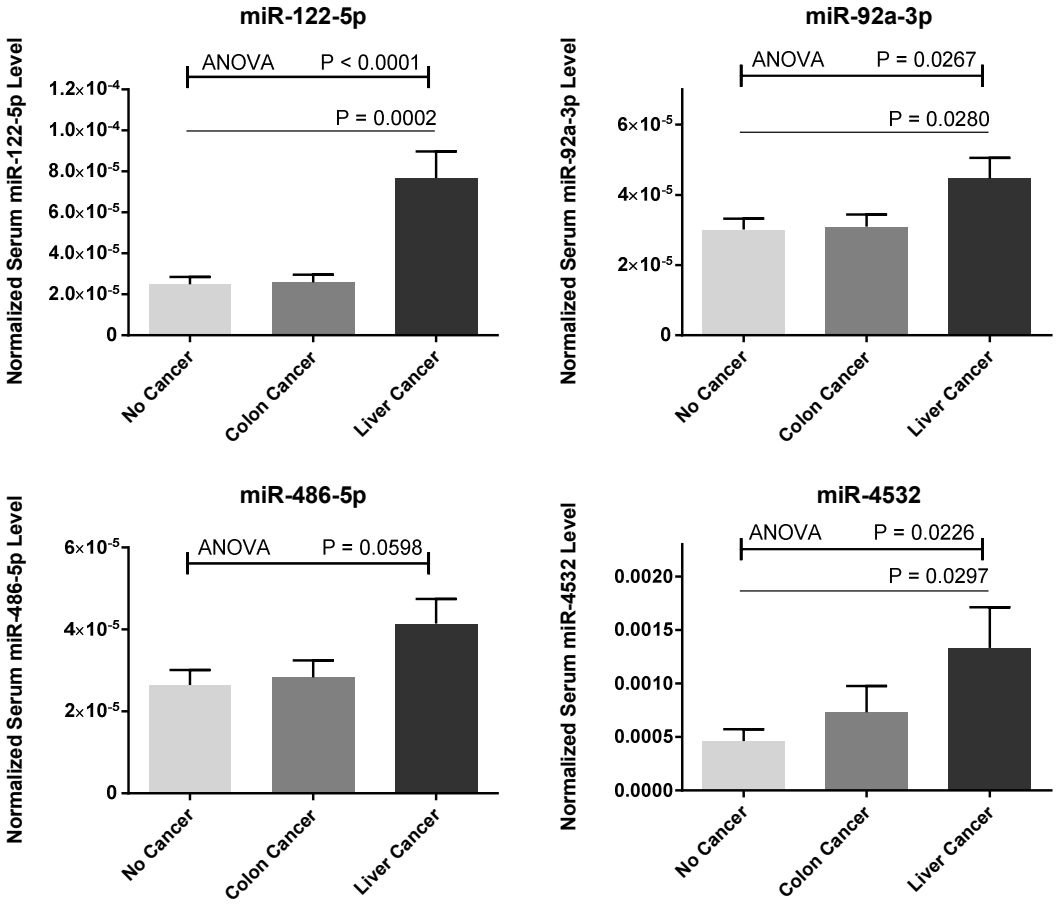

Table 1. Comparison of the selected case for the microarray study

	T2D No cancer	T2D Liver cancer	*P* value
Cases (N)	10	10	
Sex (F:M)	4 : 6	4 : 6	
Age (years)	62.40 ± 9.81	64.30 ± 7.96	0.640
Disease duration (years)	7.90 ± 5.20	7.00 ± 5.27	0.705
BMI	24.48 ± 2.95	25.02 ± 3.11	0.697
HbA1c Level (%)	7.83 ± 1.22	8.01 ± 2.91	0.860
Total cholesterol (mmol/L)	5.99 ± 0.82	5.15 ± 1.76	0.214
WBC count (10^9 cells/L)	6.58 ± 1.72	7.62 ± 4.88	0.534
Years before cancer diagnosis		3.07 ± 1.41	

Data represent in mean ± SD

Table 2. Summary of serum microRNA changes in prospective liver cancer T2D cases

Micro RNA	*P* value	Regulation	Fold change
miR-122-5p	4.46e-05	Up	3.14
miR-4454	0.0157	Up	2.10
miR-486-5p	0.0148	Up	2.10
miR-92a-3p	0.0166	Up	2.09
miR-4532	0.0124	Up	2.09
miR-320b	0.0020	Up	1.96
miR-7110-5p	0.0368	Up	1.85
miR-320c	0.0075	Up	1.78
miR-185-5p	0.0156	Up	1.76
miR-320a	0.0103	Up	1.75
miR-4674	0.0225	Up	1.73
miR-3663-3p	0.0161	Up	1.69
miR-93-5p	0.0047	Up	1.60
miR-4492	0.0275	Up	1.57
miR-320d	0.0228	Up	1.56
miR-22-3p	0.0105	Up	1.55
miR-17-5p	0.0073	Up	1.50

Table 3. Comparison of the selected case for qPCR validation

	T2D No cancer	T2D Colon cancer	T2D Liver cancer	ANOVA *P* value[1]	*P* value[2]
Cases (N)	236	139	109		
Sex (F:M)	65 : 171	63 : 76	21 : 88		
Age (years)	60.15 ± 11.23	64.68 ± 8.72	58.92 ± 10.37	0.000	0.333
Disease duration (years)	6.41 ± 6.04	7.37 ± 6.44	6.31 ± 5.56	0.264	0.889
BMI	24.46 ± 3.61	25.47 ± 3.60	23.74 ± 2.85	0.000	0.068
HbA1c Level (%)	7.92 ± 1.85	7.81 ± 1.744	7.91 ± 1.87	0.838	0.971
Total cholesterol (mmol/L)	5.25 ± 1.01	5.16 ± 1.13	4.90 ± 1.20	0.031	0.008
WBC count (10^9 cells/L)	7.37 ± 1.94	7.23 ± 1.91	6.68 ± 2.65	0.030	0.025
Years before cancer diagnosis		5.92 ± 4.72	5.17 ± 4.53	0.210	

Data represent in mean ± SD

[1] Comparison of three groups using ANOVA

[2] Comparison between cancer free T2D controls and T2D liver cancer cases using t-test

Table 4. Summary of serum microRNA levels and comparisons

	Serum level in T2D no cancer	Serum level in T2D colon cancer	Serum level in T2D liver cancer	Three groups ANOVA *P*	T2D liver cancer vs T2D no cancer *P*	T2D liver cancer vs T2D colon cancer *P*	T2D liver cancer vs No liver cancer *P*	Cancer Vs No cancer *P*
miR-122-5p	2.50e-05 ± 3.54e-06	2.58e-05 ± 3.84e-06	7.67e-05 ± 1.30e-05	< 0.0001	0.0002	0.0434	0.0002	0.0014
miR-92a-3p	3.01e-05 ± 3.04e-06	3.10e-05 ± 3.41e-06	4.47e-05 ± 5.83e-06	0.0267	0.0280	0.0002	0.0243	0.1223
miR-486-5p	2.65e-05 ± 3.60e-06	2.83e-05 ± 4.16e-06	4.14e-05 ± 6.03e-06	0.0598	0.0347	0.0761	0.0330	0.1325
miR-4532	4.60e-04 ± 1.13e-04	7.32e-04 ± 2.46e-04	1.33e-03 ± 3.80e-04	0.0226	0.0297	0.1871	0.0546	0.0291

The serum levels of miRNA detected are normalized serum level expressed in mean ±SEM.

Appendix: Financial Report

The following table is the summary of all the expense of the project.

Date	Company	Invoice No.	Items	Amount (HK$)
1/4/2016	Life technologies	16124346	primers for miRNA qPCR	2,121.6
18/5/2016	LKS core laboratory		Affymetrix miRNA 4.0 Microarray service	70,000.00
24/5/2016	Life technologies	16128067	Taqman advance miRNA cDNA synthesis kit	3,607.40
14/6/2016	Life technologies	16129514	Glycogen	1,420.00
14/6/2016	Life technologies	16129529	Taqman fast advance master mix	4,213.45
15/6/2016	Bio-Gene Tech Ltd	IN1605878	384 well plates for PCR	1,260.00
12/8/2016	Tech Dragon Ltd	TIV201608179	Oligo primers	873.60
17/8/2016	Bio-Gene Tech Ltd	IN1608156	96-well and 384-well PCR plates	2,120.00
17/8/2016	Bio-Gene Tech Ltd	IN1608197	MirX miRNA first strand synthesis kit	4,190.00
23/8/2016	Tech Dragon Ltd	TIV201608320	Oligo primers	886.60
29/8/2016	Life technologies	16134928	Taqman advance miRNA cDNA synthesis kit	14,429.60
7/9/2016	Bio-Station Ltd	BS1609010	SYBR Premix Ex Taq master mix	1,980.00
15/9/2016	Life technologies	16136205	Taqman fast advance master mix, 10 ml	7,586.25
16/9/2016	Life technologies	16136246	Taqman miRNA assay	4,243.20
20/9/2016	Life technologies	16136520	Taqman advance miRNA cDNA synthesis kit	14,429.60
20/9/2016	Tech Dragon Ltd	TIV201609240	RNA oligo	1,672.00
23/9/2016	Life technologies	16136774	Taqman Advance miRNA assays	12,729.60
7/10/2016	Life technologies	16137654	Taqman Advance miRNA assays	8,486.40
11/10/2016	Tech Dragon Ltd	TIV201610071	RNA oligo	1,558.00
25/10/2016	Bio-Gene Tech Ltd	IN1610538	P10 pipette tips	720.00
26/10/2016	Tin-Hang Tech. Ltd.	IN16-018573	SP000125, 10 mg	1,060.00
11/11/2016	Eppendorf	95032743	Multi channel pipette	6,222.40
24/11/2016	Eppendorf	95032930	Pipette tips and consumables	2,033.10
17/11/2016	Life technologies	16140711	Mitosox Red stain	2,548.00
11/1/2017	Bio-Gene Tech Ltd	IN1700429	96-well plate and sealing films	1,760.00
23/2/2017	Life technologies	17147200	Trizol and RNase free water (partial)	49.20
			Total	172,200.00