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

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).

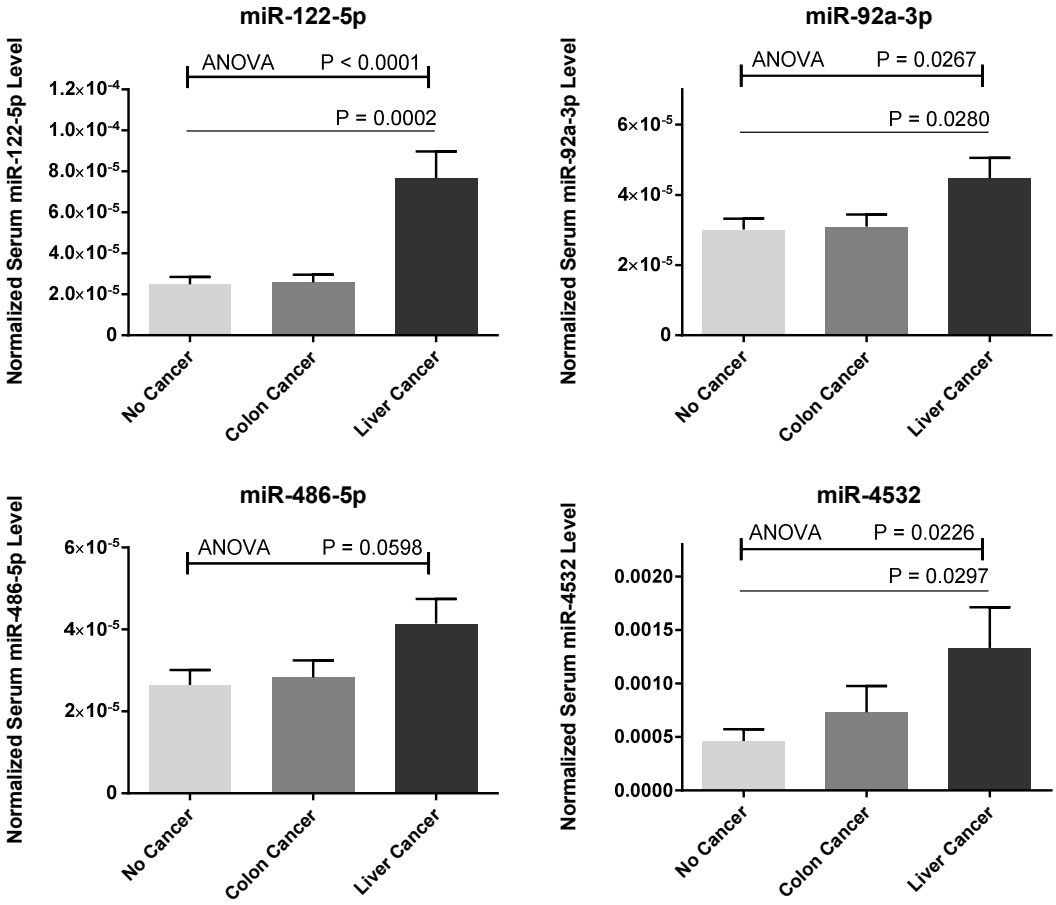

Table 1. Comparison of the selected case for the microarray study

	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.