Experimental Diabetes Research

Diabetes and Cancer: Epidemiological, Clinical, and Experimental Perspectives

Guest Editors: Chin-Hsiao Tseng, Chien-Jen Chen, and Joseph R. Landolph Jr.

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Editorial Board

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Editorial

Diabetes and Cancer: Epidemiological, Clinical, and Experimental Perspectives

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Diabetes is a major cause of death in many countries due to its increasing incidence, high prevalence, and clinical manifestation of a variety of micro- and macrovascular complications if it is not appropriately treated [1, 2]. Recent studies have shown that diabetic patients may also have a higher risk of cancer [2], the number one killer that threatens the lives of billions of people.

The clarification of the link between these two important diseases, new developments in clinical technologies and medications for the management and improvement of the survival of cancer patients in diabetic and nondiabetic individuals, and the understanding of the basic mechanisms of cancer and diabetes and their interrelationships are urgently needed for the prevention of these two diseases and the provision of better care to the patients.

In this special issue, original as well as review articles on the relationship between diabetes and cancer from the epidemiological, clinical, and experimental perspectives were invited and called for. We accepted ten papers for the publication of this special issue after careful consideration of a wide spectrum of factors including originality, novelty, and potential impacts.

In a research article, by using the National Health Insurance database from Taiwan, M. C. Hsieh et al. demonstrated a significantly higher risk of cancers involving the breast, prostate, colon, lung, liver, and pancreas in the diabetic patients. Furthermore, they disclosed that patients using insulin or sulfonylureas were more likely to develop cancer than those who used metformin. The significantly higher risk of prostate cancer associated with diabetes in the Taiwanese population in this study is consistent with a previously published paper from Taiwan [3], but is contrary to a significantly lower risk observed in Caucasian people [4]. Such a discrepancy between different ethnicities awaits further exploration.

According to the review article by N. Hara (Niigata University, Niigata, Japan), diabetes is associated with higher incidences of many cancers, including lung cancer, stomach cancer, colorectal cancer, liver cancer, and pancreatic cancer. In addition, diabetic patients may have a lower risk of overall prostate cancer, but the risk of advanced high-grade prostate cancer is actually higher in diabetic patients. Lower levels of testosterone and prostate-specific antigen observed in the diabetic patients might help explain the high-grade prostate cancer in these patients. Furthermore, androgen-deprivation therapy used for the treatment of prostate cancer may also induce insulin resistance and diabetes.

One of the mechanisms explaining the higher risk of cancer in the diabetic patients is insulin resistance. In this special issue, B. Arcidiacono et al. (Magna Græcia University of Catanzaro, Catanzaro, Italy) gave a thorough overview on the pathogenetic mechanisms linking insulin resistance and cancer risk.

The association between diabetes and thyroid cancer has been rarely studied. S. R. Shih et al. from Taiwan wrote a nice review of the literature on this topic and

proposed some potential mechanisms linking diabetes and thyroid cancer. However, future studies are required to confirm these new hypotheses. W. Y. Chiu et al. reviewed the most updated literature regarding the potential risk of thyroid cancer induced by the newly launched antidiabetic medication of glucagon-like peptide-1 receptor agonists, including exenatide and liraglutide. It is worthwhile to note that the risk may not be limited to the rare form of medullary thyroid cancer, but may also involve the more commonly seen papillary thyroid cancer.

Whether glycemic control may affect the outcome of patients with cancer is still under debate. The retrospective analyses of 265 patients with advanced breast cancer by C. Villarreal-Garza et al. from Mexico suggested that glycemic control may be an important factor related to survival in either the diabetic or nondiabetic patients. A level of >130 mg/dL is associated with a significantly higher risk of mortality.

The association between diabetes and pancreatic cancer has long been observed [5]. However, whether diabetes may induce pancreatic cancer has always been challenged because diabetes is always newonset when a diagnosis of pancreatic cancer follows [6]. A recent study suggested that new-onset diabetes with a history of dyslipidemia may predict a higher risk of pancreatic cancer [6]. The retrospective study by J. Trna et al. from Czech Republic, which evaluated the autopsy reports of 182 pancreatic cancer patients and 135 control subjects without pancreatic cancer, suggested a predominance of female sex among pancreatic cancer patients with diabetes. Although the result is preliminary, this study provided a hint for a future look into the sexual discrepancy in the risk of diabetes-related pancreatic cancer. The study by H. Yu et al. from China suggested that elevated tumor-associated antigen CA19-9 from pancreatic ductal cells may be associated with high serum total cholesterol level, impaired insulin secretion, and hyperglycemia in the diabetic patients. The findings suggest a close link between pancreatic exocrine and endocrine dysfunction and imply a potential improvement in beta-cell function with the treatment of elevated cholesterol level.

S. H. Liu and L. T. Lee from the Industrial Technology Institute, Hsinchu, Taiwan demonstrated a two-step differentiation protocol to induce mouse embryonic stem (ES) cells to differentiate into insulin-producing cells. They first treated mouse ES cells with activin to induce the mouse ES cells to differentiate into endodermal cells in a monolayer. Then, they showed that addition of nicotinamide, insulin, and laminin to the endodermal cells induced the endodermal cells to differentiate into insulin-producing cells. In a related study, G. Qing-Song et al. from Nantong University in Nantong, in China, reported a procedure for induction of insulin-producing cells from bone marrow mesenchymal stem cells (MSCs) from mice. G. Qing-Song et al. utilized a novel strategy in which multiple transcription factors—PDX-1, NeuroD1, and MafA—were transfected into mouse MSCs, and this resulted in formation of insulin-producing cells. Both the studies of S. H. Liu and L. T. Lee and the studies of G. Qing-Song et al. pave the way for more efficient beta-cell recruitment for transplantation.

In summary, the link between diabetes and cancer is an interesting issue that requires intensive exploration on different aspects including epidemiological, clinical, and experimental studies. This special issue provides a platform for the publication of some important ongoing researches and concepts. We hope that it can trigger the explosion of more fruitful progress in related fields.

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Methodology Report

Efficient Differentiation of Mouse Embryonic Stem Cells into Insulin-Producing Cells

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Embryonic stem (ES) cells are a potential source of a variety of differentiated cells for cell therapy, drug discovery, and toxicology screening. Here, we present an efficacy strategy for the differentiation of mouse ES cells into insulin-producing cells (IPCs) by a two-step differentiation protocol comprising of (i) the formation of definitive endoderm in monolayer culture by activin A, and (ii) this monolayer endoderm being induced to differentiate into IPCs by nicotinamide, insulin, and laminin. Differentiated cells can be obtained within approximately 7 days. The differentiation IPCs combined application of RT-PCR, ELISA, and immunofluorescence to characterize phenotypic and functional properties. In our study, we demonstrated that IPCs produced pancreatic transcription factors, endocrine progenitor marker, definitive endoderm, pancreatic β-cell markers, and Langerhans α and δ cells. The IPCs released insulin in a manner that was dose dependent upon the amount of glucose added. These techniques may be able to be applied to human ES cells, which would have very important ramifications for treating human disease.

1. Introduction

Human and mouse embryonic stem (ES) cells are capable of spontaneous differentiation into insulin-producing cells, among many other cell types. ES cells can be induced to preferentially differentiate into insulin-producing cells (IPCs) by changing the composition of the culture medium and causing expression of dominant transcription factor genes which are involved in pancreas development [1, 2]. In previous studies, there are two main strategies for the differentiation of ES cells into IPCs: (i) embryoid body formation and (ii) definitive endoderm formation [3–5]. Because after spontaneous differentiation the number of specifically differentiated cell types is relatively low, the application of defined differentiation factors and selection of lineage-specific progenitor cells seems to be necessary for directed differentiation of ES cells into the desired cell types [6, 7]. Differentiated cells can be obtained within approximately 33 days.

Until now, there is no report to directly induce definitive endoderm and pancreatic cells in monolayer cells at the same time. Here, we present a strategy for the differentiation of ES cells into IPCs by a two-step differentiation protocol comprising of (i) the formation of definitive endoderm in monolayer culture by activin A, and (ii) this monolayer endoderm being induced to differentiate into IPCs by nicotinamide, insulin, and laminin. The small bioorganic molecules can control cellular processes by modulation of metabolism, signal transduction pathways and gene regulation [8–18]. In our study, we demonstrated that bioorganic molecules provide key information to modulation of stem cell proliferation and differentiation at 7 days. We also combined application of the three analytical methods presented here—RT-PCR, ELISA, and immunofluorescence to characterize phenotypic and functional properties.

2. Materials and Methods

2.1. Chemicals. Leukemia inhibitory factor (LIF) was purchased from Chemicon. Mouse gelatin was purchased from BD (Becton, Dickinson and Company). Culture media and

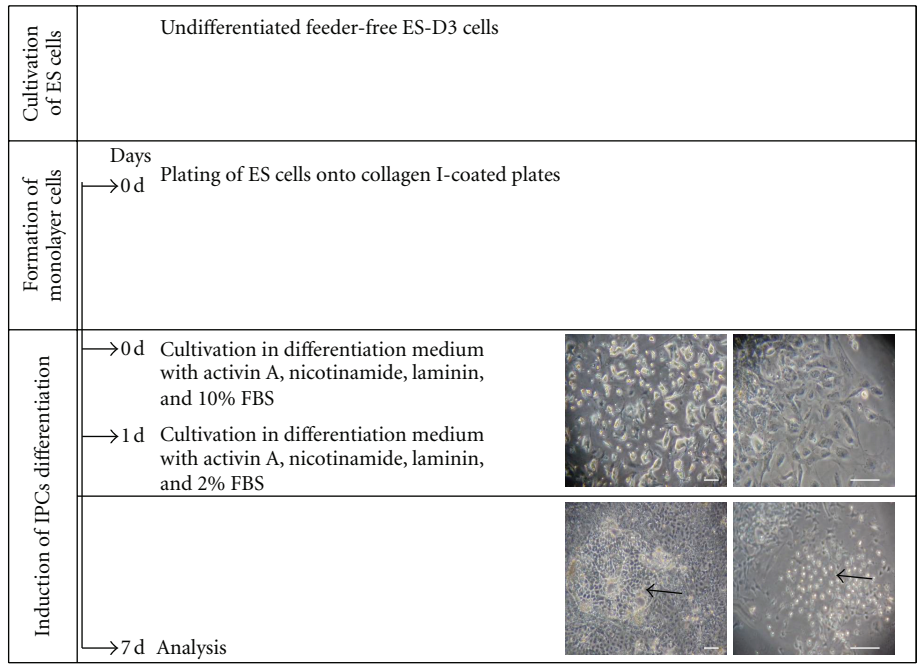

Figure 1: Schematic representations of the differentiation protocol from ES cells into insulin-producing cells. Undifferentiated feeder-free ES-D3 cells were cultured in collagen-I-coated plates and incubated in differentiation DMEM/F-12 medium supplemented with 2 mM L-glutamine, 100 μM nonessential amino acids, 10 ng/mL activin A, 10 mM nicotinamide, and 1 μg/mL laminin with 10% FBS overnight. ES-D3 cells were next exposed to DMEM/F-12 medium supplemented with 2 mM L-glutamine, 100 μM nonessential amino acids, 10 ng/mL activin A, 10 mM nicotinamide, 25 μg/mL insulin, and 1 μg/mL laminin with 2% FBS for 6 days. Differentiated ES-D3 cells formation of islet-like clusters at 7 days. Bar = 50 μm.

fetal bovine serum (FBS) were purchased from Hyclone Laboratories Inc. Activin A was purchased from R&D system. Other chemicals were purchased from Sigma-Aldrich.

2.2. Cell Culture and Differentiation. Undifferentiated ES-D3 murine embryonic stem cell lines (BCRC, 60205) were cultured on a feeder layer of mouse embryonic fibroblasts on gelatin-coated flasks in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 0.1 mM 2-mercaptoethanol supplemented with 15% fetal bovine serum (FBS), 1400 units/mL leukemia inhibitory factor (LIF) at 37°C and 5% CO_2. Subsequently, ES-D3 cells were transferred onto gelatin-coated flasks for 30 min to remove the feeder layer. ES-D3 cells were seeded at 1×10^6 cells per well to collagen-I-coated plates in DMEM/F-12 medium supplemented with 2 mM L-glutamine, 100 μM nonessential amino acids, 10 ng/mL activin A, 10 mM nicotinamide, and 1 μg/mL laminin with 10% FBS overnight. ES-D3 cells were next exposed to DMEM/F-12 medium supplemented with 2 mM L-glutamine, 100 μM nonessential amino acids, 10 ng/mL activin A, 10 mM nicotinamide, 25 μg/mL insulin, and 1 μg/mL laminin with 2% FBS for 6 days.

2.3. RNA Isolated and RT-PCR Analysis. Total RNA was isolated using PureLink Micro-to-Midi Total RNA (Invitrogen), according to the manufacturer's recommended protocol. RNA samples (1 μg/reaction) were reverse-transcribed with Superscript (Invitrogen) in the presence of oligo-dT, and the RT reaction was used for amplification with *Taq* polymerase. The resulting cDNA was amplified using specific primers. The sequences were as follows: definitive endoderm marker Sox7 (forward 5′-CCA TAG CAG AGC TCG GGG TC-3′; reverse 5′-GTG CGG AGA CAT CAG CGG AG-3′), endocrine progenitor marker Ngn3 (forward 5′-TGG CGC CTC ATC CCT TGG ATG-3′; reverse 5′-AGT CAC CCA CTT CTG CTT CG-3′), pancreatic transcription factors Pax4 (forward 5′-ACC AGA GCT TGC ACT GGA CT-3′; reverse 5′-CCC ATT TCA GCT TCT CTT GC-3′), pancreatic transcription factors Pax6 (forward 5′-TCA CAG CGG AGT GAA TCA G-3′; reverse 5′-CCC AAG CAA AGA TGG AAG-3′), pancreatic β-cell markers Insulin 1 (forward 5′-TAG TGA CCA GCT ATA ATC AGA GAC-3′; reverse 5′-CGC CAA GGT CTG AAG GTC-3′), pancreatic β-cell markers Insulin 2(forward 5′-CCC TGC TGG CCC TGC TCT T-3′; reverse 5′-AGG TCT GAA GGT CAC CTG CT-3′), Langerhans α- cells Glucagon (forward 5′-CAT TCA CAG GGC ACA TTC ACC-3′; reverse 5′-CCA GCC CAA GCA ATG AAT TCC-3′), Amylase (forward 5′-CAG GCA ATC CTG CAG GAA CAA-3′; reverse 5′-CAC TTG CGG ATA ACT GTG CCA-3′).

Langerhans δ-cells Somatostatin (forward 5′-TCG CTG CTG CCT GAG GAC CT-3′; reverse 5′-GCC AAG AAG TAC TTG GCC AGT TC-3′), β5-tubulin (forward 5′-TCA CTG TGC CTG AAC TTA CC-3′; reverse 5′-GGA ACA TAG CCG TAA ACT GC-3′). For amplification, an initial reverse

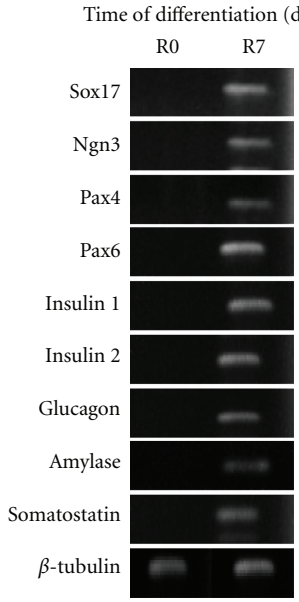

Figure 2: RT-PCR analysis of pancreatic-specific genes expression. RT-PCR analysis of undifferentiated R0 embryonic stem (ES-D3) cells and R7 cells (insulin-producing cells) at differentiation stages of 7 days. The β5-tubulin gene was used as a housekeeping-gene standard.

transcription step was followed by denaturing step (94°C for 5 minutes) and then by 30 cycles of denaturing (94°C for 30 seconds), annealing (60°C for 30 seconds), and extending (72°C for 30 seconds), followed by 7 minutes at 72°C for elongation. Glucagon and Insulin 2 annealing conditions are 55°C for 30 seconds, 65°C for 30 seconds, respectively. The PCR products produced were separated by electrophoresis on 2% agarose gel.

2.4. Immunofluorescence. The cells were fixed for 30 minutes at room temperature in 4% paraformaldehyde, then washed three times in PBS. The cells were blocked for 30 minutes in PBS plus 0.2% Triton X-100, 1% bovine serum albumin (BSA). Anti-C-peptide primary antibody (Cell Signaling Technology, Danvers, MA) was diluted 1 : 500 in PBS and incubated for 60 minutes at 37°C. The cells were rinsed three times with PBS and then incubated with fluorescence-labeled specific secondary antibody diluted in PBS with 0.5% BSA at 37°C for 45 minutes. After washing, cells were incubated with DAPI at dilution 1 : 1000 in PBS for 10 minutes.

2.5. Insulin Content. Differentiated cells were seeded at 1×10^6 cells per well in a 24 well culture plate and incubated overnight in culture media and were grown for 24 hours in DMEM/F-12 medium without insulin. Cells were then washed twice and preincubated at 37°C for 1 hour with Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 2.5 mM glucose. Cells were then incubated for 1.5 hours in KRBH buffer (contain 50 μM tolbutamide) with 2.5, 5.5, and 12.5 mM glucose. Insulin content was determined by ELISA (Mercodia).

2.6. Statistical Analysis. All data were performed in triplicate, and all experiments were repeated at least three times. Data were presented as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA, SAS 9.1.3, USA), followed by a Tukey's test to determine any significant differences. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Gene Expression and Immunofluorescence Analysis of Insulin-Producing Cells. In preliminary experiments, we found that 10 ng/mL activin A, 10 mM nicotinamide, 25 μg/mL insulin, and 1 μg/mL laminin under low serum are an ideal condition for differentiation of monolayer endoderm cells into IPCs at 7 days (shown in Figure 1).

To assess IPCs developmental changes resulting from specific modifications of culture conditions, we evaluated the expression of various genes by a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) assay. As shown in Figure 2, the results indicated that differentiated mouse ES cells expressed pancreatic transcription factors (Pax4 and Pax6), endocrine progenitor marker (Ngn3), definitive endoderm (Sox7), exocrine pancreas marker (Amylase), pancreatic β-cell markers (Insulin 1 and Insulin 2), and Langerhans α- and δ-cells (Glucagon and Somatostatin). Fluorescence micrographs also demonstrated pancreatic hormone C-peptide expressing in IPCs (shown in Figure 3(a)). After 7-day treatment with 10 ng/mL activin A, 10 mM nicotinamide, 25 μg/mL insulin, and 1 μg/mL laminin, the percentage of C-peptide expressing cells increased to 67.3 ± 2.9% (Figure 3(b)). In the time course of the next 6 days, the efficiency of C-peptide expressing does not significantly change.

3.2. Insulin Content and In Vitro Glucose-Stimulated Insulin Secretion. ES-3D cells were treated with glucose to evaluate whether IPCs released insulin in a manner that was dose-dependent upon the amount of glucose added. As shown in Figure 4(a), it can be seen that insulin is released in a manner that is dependent directly upon the amount of glucose added. At 12.5 mM glucose, the insulin released was double the amount released at .5 mM glucose, and the amount of insulin released in cells treated with 12.5 mM glucose was approximately twice that in cells treated with 2.5 mM glucose (Figure 4(a)).

In the previous studies insulin content was increased following mouse ES cells differentiation in the presence of the phosphatidylinositol 3-kinase inhibitor LY-294002. The results showed that insulin content was increased approximately 1.3-fold compared with untreated control (Figure 4(b)).

4. Conclusions

Embryonic stem (ES) cells are a potential source for insulin-producing cells (IPCs), but existing differentiation protocols are of limited efficiency. The aim has been to develop an

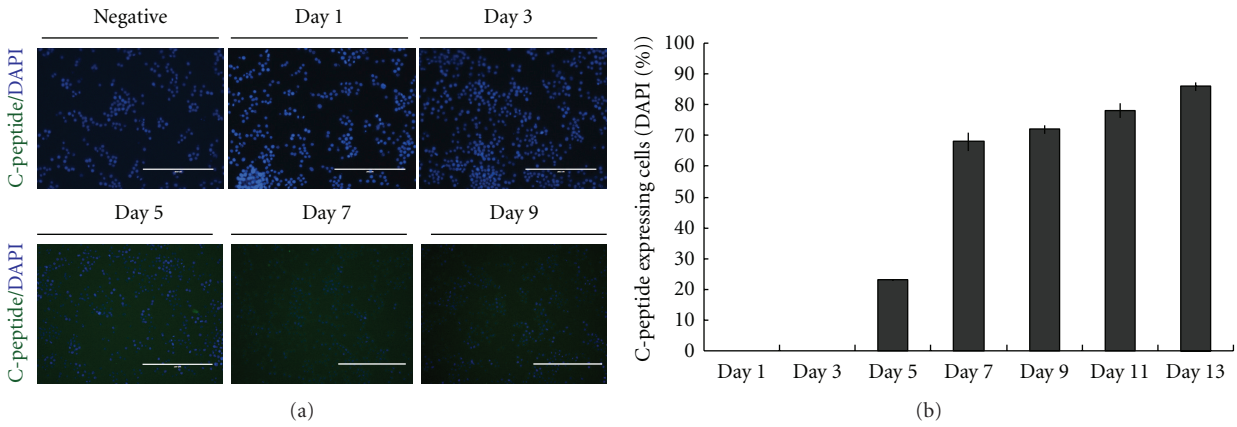

Figure 3: Immunofluorescence analysis of the C-peptide expressing cells. (a) Insulin-producing cells stain positive for C-peptide expression (C-peptide stain, green; DAPI stain, blue). RAW 264.7 (Mouse leukaemic monocyte macrophage cell line) is the negative control. Scale bars = 200 μm. (b) Experimental time course for the differentiation of ES-D3 cells to IPCs. For the quantification of IPCs, at least 10 images for each treatment were taken using an EVOS fluorescent microscope (USA). Total cell number was quantified based on DAPI nuclear staining and C-peptide expressing cells were quantified using Image J software (NIH, US). Bars represent means ± SD from three independent experiments. $^*P < 0.05$ significantly different from 7 days.

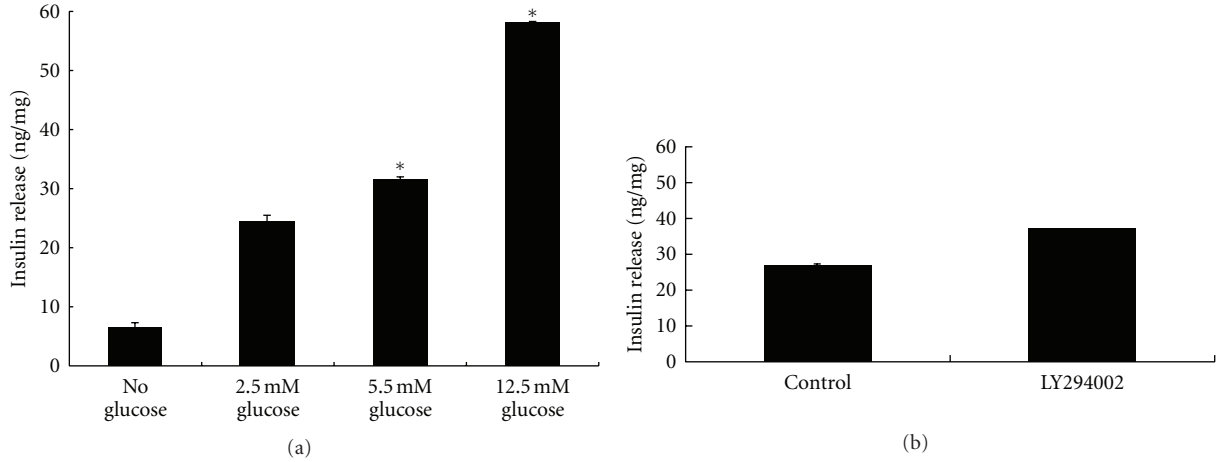

Figure 4: Intracellular insulin content. Insulin secretion was measured by ELISA and normalized to total cellular protein. (a) Insulin-producing cells were incubated with KRBH buffer containing glucose and 50 μM tolbutamide for 1.5 hours. Bars represent means ± SD from three independent experiments ($n = 3$). $^*P < 0.05$ significantly different from glucose. (b) Insulin-producing cells were incubated with 3 μM LY294002 or not (control) for 2 hours. Bars represent means ± SD from three independent experiments ($n = 3$). $^*P < 0.05$ significantly different from control.

efficient differentiation protocol, in which we could induce differentiation by small specific molecules, including activin A, laminin, nicotinamide, and insulin. Permeable small molecules can control cellular processes by modulating signal transduction pathways, gene expression, or metabolism and have been effectively used in ESC differentiation protocols. These molecules, alone or in combination with specific growth factors and hormones, will likely provide key information to design specific culture media in order to obtain IPCs [8–18]. Until now, there is no report to directly induce definitive endoderm and pancreatic cells at the same time by small specific molecules.

We here present a strategy for the differentiation of mouse ES cells into IPCs by a two-step differentiation protocol comprising of (i) the formation of definitive endoderm in monolayer culture by activin A, and (ii) this monolayer endoderm being induced to differentiate into IPCs by nicotinamide, insulin, and laminin. Activin A, a member of the transforming growth factor-β (TGF-β) superfamily, has been shown to induce endodermal differentiation of the cells of this endodermal monolayer under low serum conditions. In most of the recent studies, activin A starting from the beginning of *in vitro* differentiation monolayer can be added to cause human ES cells to differentiate into definitive endodermal cells [6]. Nicotinamide (also known as niacinamide) is a form of vitamin B3, which enhances the *in vitro* differentiation of cultured human pancreatic cells, favoring the expression of insulin, glucagon, and somatostatin [14–16]. Laminin enhances IPCs differentiation with increases in insulin and Glut2 gene expressions, proinsulin, and

insulin release in response to elevated glucose concentration [17, 18].

In this study, pancreatic β-cells (Insulin 1 and Insulin 2) and Langerhans cells' markers (Glucagon and Somatostatin) could be identified in differentiated ES cells. Fluorescence micrographs also demonstrated that pancreatic hormone C-peptide was expressed in IPCs, the percentage of C-peptide expressing cells about 67.3 ± 2.9%. Insulin content was increased in a glucose-dependent manner. After treatment with phosphatidylinositol 3-kinase inhibitor LY-294002, insulin was increased to 1.3-fold of that in untreated cells. In summary, these studies show that small molecules induce definitive endoderm and pancreatic cells in monolayer cells at the same time in the differentiation process. Our differentiation system represents an efficient protocol to direct mouse ES cells into the pancreatic lineage by generating IPCs and is applicable to further strategies for the improvement of *in vitro* differentiation into functional insulin-producing cells.

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Research Article

Impact of Diabetes and Hyperglycemia on Survival in Advanced Breast Cancer Patients

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Purpose. We examined the impact of diabetes and hyperglycemia on cancer-specific survival of patients with metastatic or recurrent breast cancer (BC). *Methods*. We performed a retrospective analysis of 265 patients with advanced BC receiving palliative chemotherapy. BC-specific mortality was compared for diabetic and nondiabetic patients as well as for patients that presented hyperglycemia during treatment. *Results*. No difference was observed between the diabetic and nondiabetic patients in terms of overall survival (OS). A difference in OS was observed between nondiabetic patients and diabetic patients who had hyperglycemia. The OS was greater in diabetic patients with proper metabolic control than diabetic patients with hyperglycemia. The risk of death was higher in patients with mean glucose levels >130 mg/dL during treatment. Several factors were associated with poor OS: tumor stage, hormone-receptor-negative tumors, HER2 negative disease, multiple metastatic sites, presence of visceral metastases, and mean glucose >130 mg/dL. *Conclusion*. Elevated glucose levels are associated with a poor outcome in diabetic and nondiabetic patients in contrast to patients with normoglycemic levels, conferring an elevated risk of death. According to these results, clinicians should monitor glucose levels during treatment for advanced breast cancer disease and take action to maintain normal glucose levels.

1. Introduction

Mexico, with a population greater than 100 million, currently has 10 million people with diabetes (types 1 and 2) [1]. Of this group, approximately 2 million are not aware of their condition, and 100,000 people will die from diabetes by the end of this year. Since 2006, breast cancer has been the leading cause of cancer mortality in Mexican women, accounting for 7.6% of female cancer-related deaths [2]. The incidence rate in 2008 was 14.63 per 100,000 women over 15 years of age, and this rate now exceeds that of cervical cancer (10.06 per 100,000 women) [2]. GLOBOCAN predictions for 2030 estimate that 24,386 women will be diagnosed with breast cancer in Mexico and that 9,778 (40.1%) will die from this disease [3]. Diabetes mellitus and breast cancer are major causes of morbidity and death in Mexico and on a global scale.

Recent research has focused attention on the effect of comorbid conditions on all-cause mortality in women with breast cancer [4]. Diabetes, characterized by hyperinsulinemia, insulin resistance, and hyperglycemia, is related to breast cancer. Elevated insulin can directly promote breast cancer cell growth and proliferation, and it can indirectly regulate a variety of factors, including insulin-like growth factors, sex hormones, and adipokines [5].

Data from multiple case-control and cohort studies and two meta-analyses report that women with a history of diabetes have a 15–20% increased risk of breast cancer

compared to women without diabetes (RR 1.15–1.20, CI 1.11–1.30) [6, 7]. In patients with breast cancer, diabetes has been associated with adverse outcomes throughout the full course of disease (i.e., initial presentation, treatment, recurrence patterns, and mortality) [7, 8]. In addition, breast cancer patients who are diabetics have a 32% increased risk of chemotherapy-related complications and a 24–61% increased risk of all-cause mortality compared to breast cancer patients without diabetes [9, 10].

An analysis of the contribution of diabetes to breast-cancer-specific mortality is difficult because of the substantial mortality attributed to diabetes alone and because diabetes is commonly associated with adverse prognostic factors specific to breast cancer. The purpose of this study was to examine the specific impact of diabetes and hyperglycemia on the cancer-specific survival of patients with metastatic or recurrent breast cancer.

2. Patients and Methods

We retrospectively reviewed the clinical records from patients diagnosed with advanced breast cancer who were treated at the National Cancer Institute of Mexico between January 2006 and December 2010. Our analysis included all of the patients with recurrent or newly diagnosed metastatic breast cancers for whom follow-up data and at least three fasting glucose measurements during treatment were available.

The following clinical and demographic data were obtained for eligible patients from their medical records: age, date of initial diagnosis, clinical stage upon initial diagnosis, pathological characteristics, and date of recurrence or progression. At the time of recurrence, the sites and number of metastatic sites, the type and number of lines of palliative treatment (with the dates of initiation and suspension for each), and any toxicity associated with treatment discontinuation were recorded.

Previous self-reported diagnoses of diabetes or its detection (fasting glucose levels $\geq$126 mg/dL) at recurrence and whether hypoglycemic treatment was received (and the treatment type) were recorded. The fasting glucose level and the body mass index (BMI) at initiation of each line of palliative therapy were obtained for diabetic and nondiabetic patients. Hyperglycemia was assigned for fasting glucose levels >130 mg/dL.

2.1. Statistical Analysis. For descriptive purposes, the continuous variables were summarized as arithmetic means with standard deviations (SDs) and medians with ranges. The categorical variables were summarized as relative frequencies, proportions, and 95% confidence intervals. Pearson Chi-square tests were used to compare the data between diabetic and nondiabetic patients and between patients with and without hyperglycemia.

Overall survival (OS) was measured from the date of advanced disease to the date of death or last followup. OS was analyzed with the Kaplan-Meier method, and comparisons among subgroups were performed with the log-rank test or the Breslow test. For breast-cancer-specific mortality, a Cox proportional hazards model was used to estimate the hazard ratio (HR) and 95% confidence intervals (95% CI). The a priori variables included in the multivariate analysis were the universally known factors associated with poor outcome (age, tumor stage, nodal status, SBR grade, hormone-receptor status, HER2 status, visceral metastatic involvement, number of metastatic sites, and diagnosis of overweight and obesity at diagnosis of recurrence), the variables of interest in this study (diagnosis of diabetes at recurrent disease, and median glucose >130 mg/dL during palliative treatment), and those variables that showed a difference in the univariate analysis with a $P < 0.01$. The SPSS software (version 17.0; SPSS, Chicago, Ill) was used for data analysis.

3. Results

A total of 265 patients receiving palliative therapy were eligible for inclusion. The median age at diagnosis was 49 years (range 22–98 years). The clinical stage at the initial breast cancer diagnosis was distributed as follows: I 10%, IIA 12%, IIB 13%, IIIA 24%, IIIB 16%, IIIC 9%, and IV 16%. Upon inclusion, 84% (225) of the study population had recurrent breast cancer, and 16% (40) had metastatic breast cancer at the initial diagnosis.

The most common histological findings were invasive ductal carcinoma and lobular carcinoma in 83% and 11% of the cases, respectively. By immunohistochemical analysis, 52.8% of the patients were hormone-receptor-positive, 25.7% had overexpression of HER2, and 24.5% were triple negative.

In patients with recurrent breast cancer, multiple metastatic sites were identified in 47% of the patients, while 53% had single-site recurrences. The site of initial recurrence was visceral in 62% of the patients and nonvisceral in 38%. The median BMI at recurrence for the study population was 27.4 kg/m^2 (SD: 4.7). Overweight and obese patients accounted for 42% and 26% of the patients, respectively. A previous diagnosis or detection of diabetes at recurrence was recorded in 40 patients (15%). Pharmacological treatment for diabetes was used by 22 patients (55%), with reported metformin use in 18 of them (45% of diabetic patients).

The differences in the clinical and pathological characteristics at the initial diagnosis or the detection of recurrence between nondiabetic and diabetic patients are shown in Table 1.

Regarding palliative treatment, there was no difference between nondiabetic and diabetic patients. Hormonal palliative treatment was delivered in a comparable proportion between the two groups: 36% versus 43% for the nondiabetic and diabetic patients, respectively ($P = 0.404$). A similar proportion of nondiabetic and diabetic patients received palliative chemotherapy during the course of recurrent disease: 93% versus 98%, respectively ($P = 0.309$). All of the patients with HER2-positive disease received trastuzumab in the palliative setting. Fifty-four percent of the nondiabetic patients received 1 or 2 lines of chemotherapy versus 59% of those with diabetes ($P = 0.590$).

Table 1: Clinical and pathological characteristics in nondiabetic and diabetic patients.

	Nondiabetics	Diabetics	Univariate (P)
Median age (Years)	48.0	52.5	0.207
T			
1	27 (12%)	4 (9%)	0.684
2	77 (34%)	12 (29%)	
3	54 (24%)	14 (35%)	
4	67 (30%)	10 (27%)	
N			
0	41 (18%)	9 (23%)	0.695
1	95 (42%)	14 (34%)	
2	56 (25%)	8 (20%)	
3	33 (15%)	9 (23%)	
Grade			
1	29 (13%)	7 (18%)	0.087
2	81 (36%)	20 (49%)	
3	115 (51%)	13 (33%)	
Hormone receptor			
Positive	112 (49%)	28 (70%)	0.018
Negative	113 (51%)	12 (30%)	
HER2			
Positive	60 (27%)	5 (13%)	0.079
Negative	165 (73%)	35 (87%)	
Triple-negative			
Positive	59 (26%)	7 (18%)	0.262
Negative	166 (74%)	33 (82%)	
Metastatic site			
Single	122 (54%)	18 (45%)	0.306
Multiple	103 (46%)	22 (55%)	
Type of metastases			
Nonvisceral	86 (38%)	16 (40%)	0.790
Visceral	139 (62%)	24 (60%)	
Median BMI at recurrence	26.8	27.5	0.359

T: tumor stage according to TNM; N: nodal stage according to TNM; BMI: body mass index.

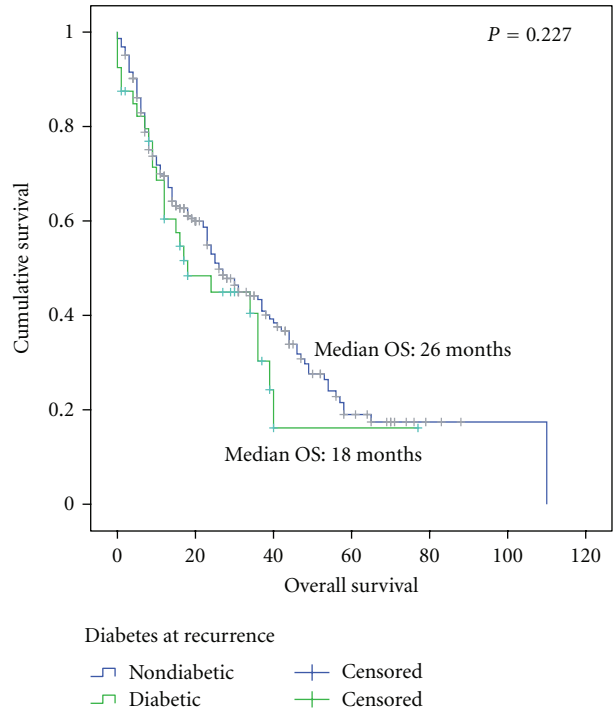

Figure 1: Overall survival since diagnosis of advanced disease between nondiabetic ($n = 225$) and diabetic ($n = 40$) patients.

No difference was identified in the proportion of nondiabetic and diabetic patients who experienced toxicity that lead to the suspension of treatment. Grade 3/4 toxicity during palliative treatment was experienced by 14% and 19%, respectively, of the nondiabetic and diabetic patients, which was not significantly different ($P = 0.253$).

All of the deaths were related to breast cancer. The median OS for the entire group was 26.0 months since the diagnosis of recurrence. According to the hormonal and HER2 receptor status, differences were observed as expected for the entire group of patients. For the hormone receptor-positive breast cancer patients, the OS was significantly longer than their counterparts (37.0 versus 18.0 months, respectively, $P < 0.001$). In the triple negative group, the OS was lower compared to the non-triple negative patients (15.0 versus 31.0 months, respectively, $P = 0.005$). As for HER2 status, there was no difference between the patients with HER2-positive and HER2-negative breast cancer (26.0 versus 24.0 months, respectively, $P = 0.824$).

For the OS analysis, no difference was observed between the nondiabetic and diabetic patients in terms of OS (26.0 versus 18.0 months, respectively, $P = 0.227$) (Figure 1). However, a statistically significant difference in OS was observed between patients without diabetes and diabetic patients who had hyperglycemia (average fasting glucose level >130 mg/dL), with an OS of 36.0 months versus 12.0 months ($P = 0.003$), respectively (Figure 2).

The OS in diabetic patients with proper metabolic control (average fasting glucose level <130 mg/dL) ($n = 24$) compared to the OS in diabetic patients with hyperglycemia ($n = 16$) was shown to be superior (OS not reached versus 12.0 months, respectively, $P = 0.01$) (Figure 3). The use of metformin showed a nonsignificant benefit in OS in diabetic patients (17.0 months versus 10.2 months metformin-receiving and non-metformin-receiving patients, resp., $P = 0.371$).

For the entire cohort (diabetic or nondiabetic), patients with mean glucose levels >130 mg/dL during the administration of palliative treatment had a poorer OS compared to patients who did not experience hyperglycemia (OS 27.0 versus 12.0 months, resp., $P = 0.023$) (Figure 4).

Hyperglycemia (fasting glucose level >130 mg/dL) was identified in 32 patients (14.24%) of the nondiabetic population at some point during their treatment. Including the diabetic and nondiabetic subgroups, 60 patients were identified with at least one fasting glucose measurement greater than 130 mg/dL during their palliative treatment.

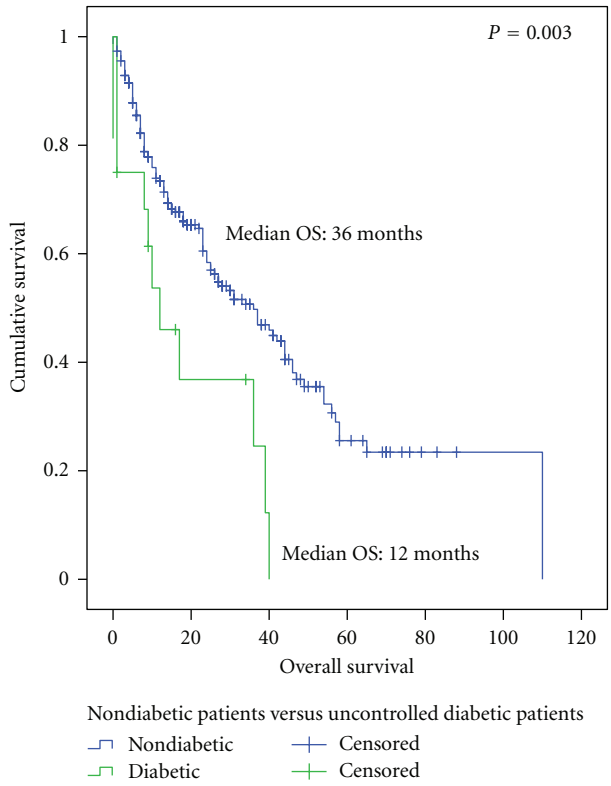

Figure 2: Overall survival since diagnosis of advanced disease between nondiabetic patients ($n = 225$) and uncontrolled diabetic patients (mean glucose >130 mg/dL) ($n = 16$).

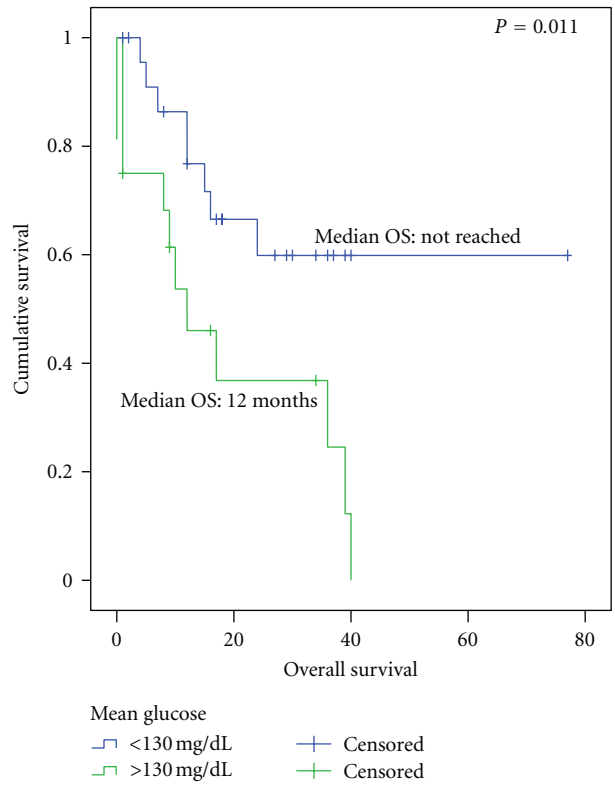

Figure 3: Overall survival since the diagnosis of advanced disease in diabetic patients according to mean glucose >130: normoglycemic ($n = 24$) versus hyperglycemic ($n = 16$).

Comparing patients who never experienced hyperglycemia ($n = 205$) to this group, a trend towards a lower OS was observed for patients with hyperglycemia, although this difference did not reached statistical significance (OS 27.0 versus 17.0 months, resp., $P = 0.07$) (Figure 5).

The OS was compared between these two groups according to hormonal and HER2 receptor status. For the hormone-receptor-negative subgroup, there was no difference in the OS between the diabetic and nondiabetic patients. However, for the hormone-receptor-positive subgroup, the nondiabetic subgroup had a significantly longer OS than the diabetic subgroup (41.0 versus 24.0 months, resp., $P = 0.035$). Similar results were found for the HER2 receptor status. No difference was observed in the HER2-negative patients between the diabetic and nondiabetic patients. For the HER2-positive subgroup, a longer OS was observed in the nondiabetic patients than to their counterparts (27.0 versus 9.0 months, resp., $P = 0.062$). When triple-negative status was considered, there was no difference in the OS for the diabetic and nondiabetic patients in either the triple negative or the non-triple negative subgroups.

The median age at breast cancer diagnosis was 49 for nondiabetic and diabetic patients. The patients were dichotomized according to the median age value: patients <49 and ≥49 years old. No difference was observed among young patients regarding the diagnosis of diabetes. However, for patients older than 49 years, nondiabetic patients had a longer OS compared to diabetic patients (37.0 versus 15.0 months, resp., $P = 0.043$).

For differences among nondiabetic and diabetic patients according to weight and obesity, patients were dichotomized based on BMIs greater than or lower than 25. No significant difference in OS was observed between the groups.

Table 2 shows data on the OS according to clinical and pathological variables analyzed by univariate and multivariate analyses. In the multivariate analysis, several factors were associated with poor OS, including tumor stage 3/4 (HR 2.7, 95% CI 1.7–4.4, $P < 0.001$), hormone-receptor-negative tumors (HR 0.2, 95% CI 0.1–0.6, $P = 0.003$), HER2 negative disease (HR 0.3, 95% CI 0.1–0.8, $P = 0.015$), multiple metastatic sites (HR 1.6, 95% CI 1.0–2.4, $P = 0.047$), presence of visceral metastases (HR 1.6, 95% CI 1.0–2.5, $P = 0.042$), and mean glucose >130 mg/dL (HR 2.8, 95% CI 1.1–7.3, $P = 0.034$).

4. Discussion

Despite the growing body of evidence indicating that diabetes predicts a poor prognosis after a diagnosis of breast cancer, whether a threshold of glycemic status at which the risk for a poor prognosis significantly increases remains unknown. In our cohort of patients with advanced breast cancer, a diagnosis of diabetes was not associated with a poor outcome. However, when uncontrolled diabetic

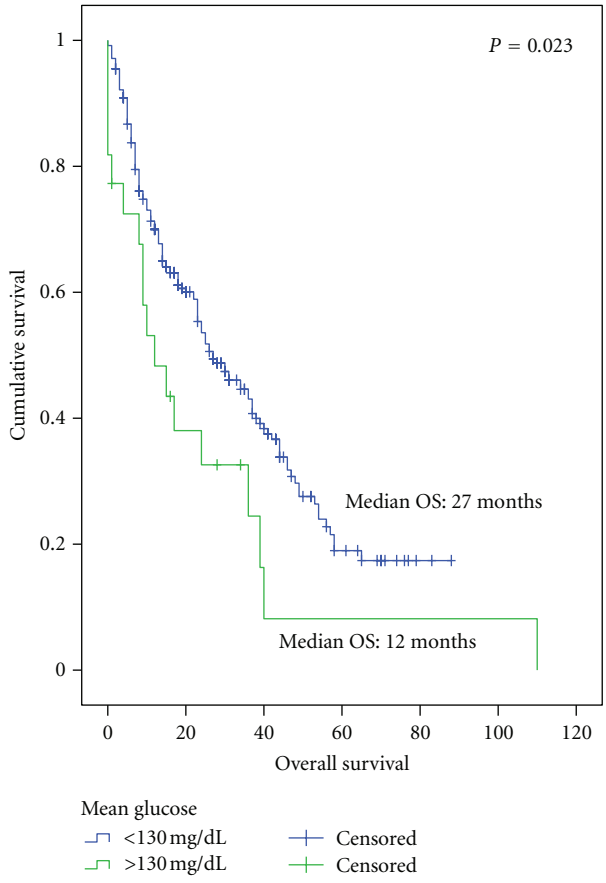

Figure 4: Overall survival since the diagnosis of advanced disease in all of the cohorts according to mean glucose ≤130 ($n = 243$) versus glucose >130 ($n = 22$).

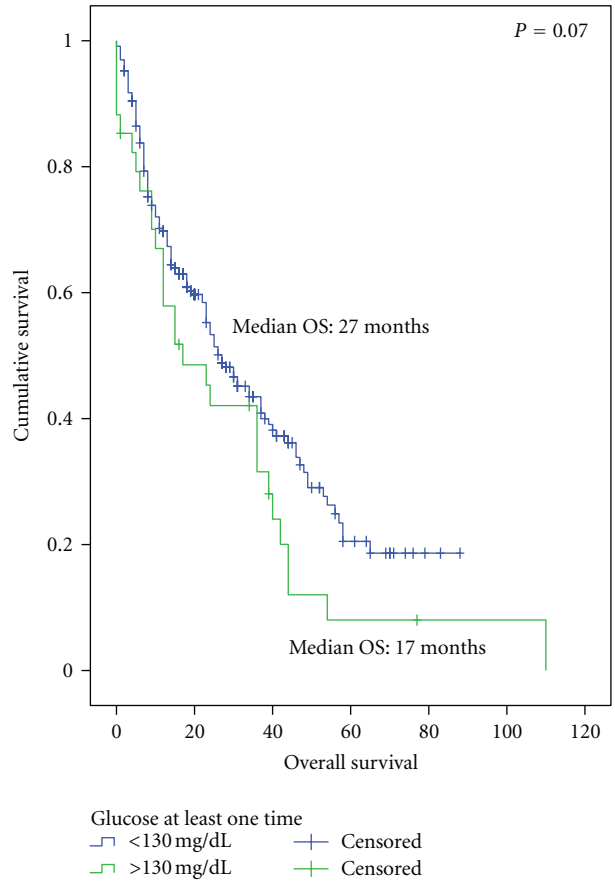

Figure 5: Overall survival since the diagnosis of advanced disease in patients that presented a glucose level >130 mg/dL at least one time during palliative treatment: normoglycemic ($n = 205$) versus glucose >130 ($n = 60$).

patients were compared to nondiabetic patients, there was a significant difference in OS, suggesting that poor control of diabetes has a negative impact in patients with metastatic breast cancer receiving palliative treatment. Moreover, diabetic patients with hyperglycemia had a worse prognosis compared to diabetic patients with normal glucose levels.

Hyperglycemia was identified in 14% of nondiabetics at some point while receiving palliative treatment. For patients in either the diabetic or nondiabetic subgroups that experienced hyperglycemia during treatment or who had a mean glucose level greater than 130 mg/dL, a worse outcome was observed compared to normoglycemic patients, with a HR of 1.5 and HR of 2.04 for death, respectively. Erickson et al. recently reported that chronic hyperglycemia (defined as hemoglobin A1C levels ≥6.5%) was independently associated with a statistically significant higher risk of all-cause mortality in early-stage breast cancer survivors, independently of a self-reported diagnosis of diabetes [11].

Hyperglycemia may directly influence breast cancer progression and outcomes via several mechanisms, including pathways mediated by high levels of insulin and insulin-like growth factors, sex hormones, and inflammatory markers [11]. Hyperinsulinemia may augment cell proliferation and survival [12, 13].

Diabetes has been associated with higher all-cause mortality in women with breast cancer [8]. Several reasons related to the diagnosis of breast cancer in diabetic patients may explain the worse outcomes observed in this group. Women with diabetes may experience a delay in diagnosis, causing them to present with more advanced breast cancer. Because of the concurrent treatment of the chronic diseases associated with diabetes, patients may not undergo routine screening for breast cancer [14]. Furthermore, women with diabetes may receive less aggressive treatment, including chemotherapy, radiotherapy, and/or surgery [8, 15]. The administration of less aggressive treatment may be related to their underlying comorbidities precluding treatment options or a perceived risk of therapy-related toxicity in patients with diabetes [8]. Additionally, women with preexisting diabetes may have a greater risk of chemotherapy-related toxicity, such as infection, fever, and neutropenia. Such risks might explain and justify the use of less aggressive treatments [16, 17].

The measurement of substantial mortality attributed solely to diabetes is difficult to assess because diabetes is commonly associated with adverse prognostic factors specific to breast-cancer and other comorbidities. Fleming et al. [18]

Table 2: Univariate and multivariate analyses according to overall survival.

Variable	Overall survival (median, months)	*P*	HR (95% CI)	*P*
Age (years)			1.2	
Age < 49	26	0.301	(0.8–1.9)	0.303
Age 49	26			
Tumor stage				
1-2	38	**<0.001**	**2.7 (1.7–4.4)**	**<0.001**
3-4	17			
Nodes				
Negative	38	0.276	0.6 (0.4–1.1)	0.091
Positive	24			
Grade				
1-2	39	**0.004**	1.5 (0.9–2.2)	0.083
3	20			
Hormone receptor				
Positive	37	**<0.001**	**0.2 (0.1–0.6)**	**0.003**
Negative	18			
HER2				
Positive	26	0.824	**0.3 (0.1–0.8)**	**0.015**
Negative	24			
Triple-negative				
Positive	15	**0.005**	0.4 (0.1–1.1)	0.072
Negative	31			
Metastatic site				
Single	38	**0.018**	**1.6 (1.0–2.4)**	**0.047**
Multiple	24			
Type of metastases				
Nonvisceral	38	**0.019**	**1.6 (1.0–2.5)**	**0.042**
Visceral	23			
Overweight or obesity at recurrence				
Yes	31	0.310	0.9 (0.6–1.4)	0.680
No	23			
Diabetes at recurrence				
No	26	0.227	0.9 (0.4–1.9)	0.709
Yes	18			
Mean glucose >130				
No	27	**0.023**	**2.8 (1.1–7.3)**	**0.034**
Yes	12			

HR: Hazard ratio.

observed no increase in breast-cancer-specific mortality in patients with diabetes, whereas Srokowski et al. [10] identified increased breast cancer-specific mortality only in patients receiving chemotherapy. The interpretation of these results are further confounded by the findings of Lipscombe et al. [19], who reported a similar mortality in diabetic patients with and without breast cancer. In our cohort, only advanced breast cancer patients were included, thus eliminating possible confounders in prediagnosis delay, clinical prognostic factors or management at initial diagnosis. In addition, all of the patients received palliative treatment for advanced disease, and the deaths were due to breast-cancer-specific causes. Thus, deaths related to other comorbidities did not influence the patients' outcomes. None of the patients abandoned treatment due to treatment-related toxicity, so this risk did not contribute to survival outcome.

Population studies suggest that metformin decreases the incidence of cancer and cancer-related mortality in diabetic patients [20, 21]. More recently, a retrospective study of patients who received neoadjuvant chemotherapy for breast cancer showed that diabetic cancer patients receiving metformin during their neoadjuvant chemotherapy had a higher pathological complete response rate than diabetic patients not receiving metformin (24% versus 8%, resp., $P = 0.007$) [22]. The antineoplastic effects of metformin in breast

cancer are supported by a biological rationale involving important factors associated with breast cancer prognosis. In our study, diabetic patients that received treatment with metformin had a longer OS compared to diabetics with no such treatment. This difference did not reach statistical significance, which was most likely due to the small sample of patients that were managed with this drug. Currently, two-phase I-II trials are ongoing to evaluate the benefit of adding metformin to the treatment of metastatic breast cancer disease. A related phase III trial is being conducted in an adjuvant setting.

Frequently, breast cancer patients treated with chemotherapy receive steroidal agents to avoid or reduce specific adverse events. The hyperglycemia observed in nondiabetic patients while on palliative treatment might be a secondary effect from the use of steroids. Due to the limitations of this study from its retrospective design, we could not associate the rate of hyperglycemia with the use of this type of drug class.

In our study, we observed a worse OS among diabetic patients older than 49 years, a difference that was not observed in younger patients. This finding is consistent with a meta-analysis that showed a stronger association (overall summary RR 1.19, 95% CI 1.15–1.23) among postmenopausal women or among women of postmenopausal age and no significant association for premenopausal women or women of premenopausal age [6]. However, a study in Taiwanese breast cancer patients demonstrated that although a higher risk of breast cancer mortality in diabetic patients occurred in all of the age groups, the magnitude of the risk was largest in the younger age group of 25–54 years [23]. One possible explanation for the discrepancies could be the ethnicities of the study populations because none of the studies were analyzed according to the menopausal status as in the meta-analysis that enrolled Asian women [23].

In conclusion, independent of the cause of hyperglycemia, elevated glucose levels are associated with a poor outcome in diabetic and nondiabetic patients in contrast to patients with normoglycemic levels, conferring an elevated risk of death. According to these results, clinicians should monitor glucose levels during treatment for advanced breast cancer disease and should take action to maintain normal glucose levels.

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Research Article

Diabetes Mellitus in Pancreatic Cancer Patients in the Czech Republic: Sex Differences

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Aims. The prevalence of diabetes mellitus in pancreatic cancer patients and control subjects was compared. *Methods*. Retrospective evaluation of 182 pancreatic cancer patients and 135 controls. The presence of diabetes was evaluated and the time period between the diagnosis of diabetes and pancreatic cancer was assessed. A subanalysis based on patient sex was conducted. *Results*. Diabetes mellitus was present in 64 patients (35.2%) in pancreatic cancer group and in 27 patients (20.0%) in control group ($\chi^2 = 8.709$; $P = 0.003$). In 18 patients (28.1% of diabetic pancreatic cancer patients) diabetes was new-onset. Diabetes was new-onset in 23.3% of females compared to 38.1% of males ($\chi^2 = 1.537$; $P = 0.215$). The overall prevalence of diabetes was significantly higher among female pancreatic cancer patients (25% versus 43.9%; $\chi^2 = 7.070$, $P = 0.008$), while diabetes prevalence was equally represented in the control group patients (22.1% versus 17.2%; $\chi^2 = 0.484$, $P = 0.487$). *Conclusion*. The prevalence of diabetes mellitus in study group of pancreatic cancer patients was significantly higher when compared to control group. Pancreatic cancer patients with diabetes were predominantly females, while diabetes was equally prevalent among sexes in the control group. Therefore, patient sex may play important role in the risk stratification.

1. Background

Pancreatic cancer (PC) represents the fourth leading cause of cancer death with an increasing incidence. Despite the efforts for the improvement of diagnostics and therapy, the prognosis of patients with PC remains dismal, with an overall 5-year survival rate of approximately 5% [1–3]. One of the primary causes for this unfavorable situation is the long asymptomatic course of the disease, so when the disease is discovered, it is usually in the advanced stage and curative surgery is typically impossible [4]. Invasiveness, early metastazing, and resistance to radio- and chemotherapy are additional reasons for the disappointing prognosis.

In view of these facts, current pancreatologic research focuses on the identification of risk factors and the determination of high-risk patient groups to increase the potential for screening and early diagnosis of PC. Currently there are screening programs for patients with several genetic disorders, including hereditary chronic pancreatitis, who are at a higher risk of progression to PC [5]. But high risk groups in the general population are still not well defined.

One of the long debated factors linked to pancreatic cancer is diabetes mellitus (DM). The association of DM and PC is complex and not completely understood. Whether DM is a risk factor for the development of PC or its first symptom remains a matter of research and debate [6–10]. However, subjects with new-onset DM have been shown to have a higher than expected likelihood of having PC [11, 12] and screening of new-onset diabetics has been proposed as a strategy to improve unfavorable outcomes of PC treatment [13].

The primary aim of our study was to retrospectively establish the prevalence of DM in the group of PC patients autopsied in our institution, to record the chronology of the diagnoses and compare the results with the control group.

2. Methods

We retrospectively evaluated autopsy reports and related documentation including final hospital release forms of 182 consecutive PC patients who died between 2001 and 2005 in the South Moravian region of the Czech Republic. The study was restricted to patients diagnosed with PC prior to their death based on clinical presentation, imaging methods, and/or results of surgery and autopsy with corresponding diagnosis in the Department of Pathology of University Hospital Brno. As a control group, 135 patients of comparable age and sex distribution who died of non pancreas-related disease during the same time interval were randomly selected from the database based on alphabetical search. Retrospective studies of unidentified data of deceased patients who did not decline research participation are generally allowed by our institution without an additional approval procedure. All the autopsies and microscopic confirmations of PC diagnosis were conducted by experienced pathologists. Presence of DM, length of its duration, and its duration prior to the diagnosis of PC, as well as symptoms leading to the investigation, were based on data from the medical records. Chi-square analyses were conducted to determine whether frequency of DM diagnosis differed significantly between PC and control groups. In addition, a subanalysis based on sex status of the persons was conducted.

3. Results

The data of 182 patients (84 males, 98 females) who died due to PC and 135 controls (77 males, 58 females) were analyzed. The mean age of the patient population was 68.7 ± 10.7 years (67.6 for males, 69.6 for females) and 71.0 ± 11.7 years for the controls (69.2 for males, 71.7 for females). The difference was statistically insignificant ($P = 0.066$). The youngest enrolled person was 34 years old, the oldest was 96.

The clinical symptoms leading to medical investigation and PC diagnosis, including their length, are summarized in Table 1. The symptoms preceded PC on average only by weeks to months.

The causes of death from medical documentation of patients in the control group are summarized in Table 2.

DM was present in 64 PC patients (35.2%); 21 were male (25% of male population) and 43 were female (43.9% of female population). Therefore, the prevalence of DM was significantly higher among female PC patients ($\chi^2 = 7.070$; $P = 0.008$).

DM was present in 27 patients (20.0%) in the control group; 17 were male (22.1%) and 10 were female (17.2%). Therefore, the significant difference in DM prevalence among the sexes seen in the PC patient group was not found in the control group ($\chi^2 = 0.484$; $P = 0.487$).

In global, chi-square analyses revealed a statistically significant difference in the prevalence of DM among patients comprising the PC and control group ($\chi^2 = 8.709$; $P = 0.003$).

The average duration of DM prior to the diagnosis of PC was 8.2 years (range of 1 to 23 years). In 18 PC patients (28.1% of the PC group; 8 male, 10 female), DM was diagnosed less than 3 years prior to PC diagnosis. In regard to sex differences, DM was a new-onset diagnosis in 23.3% of diabetic females compared to 38.1% new-onset male diabetics ($\chi^2 = 1.537$; $P = 0.215$). Thus, the trend in the difference in onset of DM among the sexes in the PC group did not reach statistical significance, partially because of small sample size. The results are summarized in Table 3.

4. Discussion

PC is a disease with a dismal prognosis that is characterized by a typically long, asymptomatic course and with limited detection of early curative stages [14]. The majority of patients (>85%) have unresectable disease by the time disease-specific symptoms develop and the diagnosis is made [4]. Correspondingly, the symptoms in our PC patients preceded the PC diagnosis on average only by weeks to months (Table 1). Therefore, in order to detect surgically treatable stages of PC, asymptomatic individuals must be screened [13].

The incidence of PC is too low for cost-effective screening within the general population. For effective and economical screening, it is necessary to establish risk factors and screen persons at high risk for PC development [13]. Currently, there are screening programs for patients with several genetic disorders, who are at higher risk for PC progression [5, 15–19]. However, high risk groups in the general population have not yet been well defined [20].

Diabetes mellitus is a factor that has long been discussed in relation to PC. While a meta-analysis published in 2011 confirmed the overall increased risk of PC among diabetics (RR = 1.94), subgroup analysis revealed that the relative risk of PC was correlated negatively with the duration of DM, with the highest risk of PC found among patients diagnosed with DM within 1 year (RRs = 5.38) [21]. It is reasonably believed that in some individuals a new-onset DM may be the first symptom of an otherwise asymptomatic PC [10, 11, 22, 23].

The prevalence of DM among PC patients in this study was 35.2%. In comparison, diabetes was present in 20.0% of patients in the control group. This difference was statistically significant, and it supports the role of DM in PC. These results are consistent with other reports in the literature, which have documented rates of DM in PC patients ranging from 8.5%–40% [10, 11, 22, 23].

Our results (DM in PC patients 35.2% versus controls 20.0%) are surprisingly similar to the results of a recent study by Chari et al. [23], who found DM in 40.2% of patients and in 19.2% of controls using fasting blood glucose levels and/or antidiabetic medication for DM identification. They report that prevalence of DM was similar in PC patients and controls 3 years before PC diagnosis. A continuous increase of DM prevalence was observed as time approached PC diagnosis in the patient group, while it remained stable within the control group.

The prevalence rate reported in this study is unusually high for retrospective autopsy methodologies relying on medical records; such studies typically report DM in less than 20% of PC patients [12, 24]. This may be because

Table 1: Symptoms preceding the diagnosis of PC.

Symptoms leading to investigation	Present in number of patients (%)	Average length prior PC diagnosis	Shortest and longest interval
Weight loss	75 (41.2%)	3.1 month	1 week–1 year
Painless icterus	50 (27.5%)	1.5 week	2 days–2 month
Back or abdominal pain	44 (24.2%)	2.2 month	1 week–1 year
Dyspepsia, loss of appetite, nausea, vomiting	41 (22.5%)	2.6 month	1 week–1 year
Ascites	14 (7.7%)	2.3 week	1 week–1 month

Table 2: Causes of death of control group patients.

Cause of death according to medical records	Number of patients (%)
Stroke, intracerebral hemorrhage	18 (13.4%)
Cardiovascular disease	52 (38.8%)
Cancer (not PC)	31(23.1%)
Terminal bronchopneumonia	21 (15.7%)
Other	12 (9.0%)

more than 25% of DM remains undiagnosed and does not enter medical records [25, 26]. In the case of PC, the prevalence of undiagnosed diabetes is believed to be even higher (approximately 50%) because the PC manifests before the DM can become symptomatic and diagnosed [11]. While this may be the situation in a health care system based on personal freedom, such as those in the United States, we believe that the system of annual preventive medical check-ups that used to be organized and enforced by law in the Czech Republic may lead to better detection of conditions like DM in today's generations of seniors.

In cohort studies, the prevalence of DM in the general population over 60 years of age has been reported as 21% in Poland [27] and 16.9% in the USA [28], both of which are comparable with the prevalence of 20.0% in our control group. This lends support to the accuracy of our retrospective results when compared with prospective studies.

DM was more common in female PC patients than in male PC patients (43.9% and 17.2%, respectively) and a difference of this magnitude was not seen among the control group patients. To our knowledge, no study thus far has compared DM prevalence among the sexes in patient and control groups. The sex status based comparison of DM prevalence among PC patients alone has been evaluated by several studies. In agreement with our results, Souza et al. found a higher prevalence of DM among female patients in their retrospective study of 151 PC patients [29]. The reason for this difference is unclear, but according to this study, it cannot be explained by higher BMI in women, as BMI was comparable among the sexes. However, Pannala's study provided opposite results, with males exhibiting DM more frequently than females [22]. Additionally, other studies did not find an increased risk of DM associated with PC in women versus men [30]. However, female diabetics have been suggested to have a higher risk of PC development [6]. This issue deserves further research, as a sex status might represent an additional risk factor useful for diabetics risk stratification with long-standing female diabetics and new-onset male diabetics being at a higher risk of PC developement.

New-onset DM (less than 3 years prior to PC) was diagnosed in 28.1% of our PC patients with diabetes. The percentage of patients with differently defined new-onset diabetes is reported to be 52–100% of all the diabetic PC patients [11, 31]. Our data did not suggest such a high prevalence, which may be partially explained by our retrospective methodology; in our opinion, a retrospective method may prove more reliable in detecting total numbers than detail temporal associations [32]. Additionally, the heterogeneity of new-onset DM definitions in the published literature often makes the comparison difficult.

Patients with newly diagnosed DM are at a substantially increased risk of PC appearnce during the first few years of followup [10, 11, 33]. In the majority of the studies, including the current study, the patients diagnosed with DM did not present with any other symptoms of PC; therefore, DM may be considered the first symptom of the cancer [23]. Our study was not capable of unequivocally verifying this hypothesis, but the short duration of symptoms leading to diagnosis of advanced PC compared to duration of DM is suggestive. Moreover, PC appears to be resectable in most of the patients at the time of DM onset and therefore, this situation might represent a valuable tool in screening for PC [34, 35].

However, PC diagnosed within 3 years of the DM diagnosis represents only about 1% of newly diagnosed diabetics over 50 years of age [11]. Thus, not even this group can be considered a high risk group and tested with sophisticated modern diagnostic methods. Therefore, researchers have worked on criteria distinguishing between DM caused by PC and "common" type 2 DM [11, 12, 32]. Several studies have screened patients with new-onset DM and defined clinical symptoms resulting in frequent diagnosis of PC [12, 36]. Unfortunately, the resectability rates were low, likely because clinical PC symptoms were used to identify the subjects for screening and the short duration of cancer related symptoms prior to the diagnosis of advanced PC was demonstrated by us as well as by others [23]. Pannala et al. conclude, that before the onset of PC symptoms, the clinical profile of PC-associated DM is not very different from that of the patient with type 2 DM and does not help

Table 3: DM in PC patients and control group.

	PC patients	Control group	
Population (M/F)	182 (84/98)	135 (77/58)	
Mean age (years) (M/F)	68.7 ± 10.7 (67.6/69.6)	71.0 ± 11.7 (69.2/71.7)	$P = 0.066$
DM	64 (35.2%)	27 (20.0%)	$\chi^2 = 8.709$; $P = 0.003$
DM (M/F)	21 (25%)/43 (43.9%)	17 (22.1%)/10 (17.2%)	
	$\chi^2 = 7.070$; $P = 0.008$	$\chi^2 = 0.484$; $P = 0.487$	
New-onset DM (M/F)*	8 (38.1%)/10 (23.3%)	3 (17.6%)/2 (20%)	
	$\chi^2 = 1.537$; $P = 0.215$	$\chi^2 = 0.023$; $P = 0.879$	

* DM diagnosed less than 3 years prior to PC diagnosis.

to distinguish between these two forms of DM [22]. Further research is necessary to clarify this controversial issue.

The differentiation between the two types of DM for PC would be simplified by assessment of putative factors produced specifically by PC cells, but their clinical use is usually disappointing [37]. A screening of patients with the combination of new-onset DM and positive family history of PC seems to be useful for detecting of early or premalignant changes [38].

Our study's main limitations include its retrospective nature and inability to conclusively answer epidemiological questions due to a patient group versus control group comparison design. However, the prevalence of pancreatic cancer in the population of the Czech Republic was previously established by our research group as 19.1 per 100,000 males and 18.2 per 100,000 females in 2007. In the time period of 1989–2005, the prevalence of pancreatic cancer increased by 45.9% in males and by 119.1% in females [39, 40]. Similarly, our research was not focused on determining the percentage of clinically undiagnosed PC among autopsied patients.

Our study's strengths include the data comparison of well-documented groups in which the diagnoses were verified by autopsy. Our study contributes a new perspective with findings of a significantly higher prevalence of DM among female PC patients while DM prevalence was equally represented among the sexes in the control group. The reason for this is not fully understood and this study suggests that there is a potential for future research in this area. Additionally, the verification of the data gathered mostly on the Northern American continent on the population of the Czech Republic is valuable because it decreases the risk of population selection bias.

5. Conclusions

As a first-of-its-kind study from Central/Eastern Europe, we provided results of a high prevalence of DM among PC patients which was new-onset in a significant portion of patients. DM was predominant in females among the PC patients, while the DM prevalence was similar among sexes in the control group. The reason for this is unknown and deserves further research, as it might be useful for risk stratification. In general, patients with new-onset DM, especially those presenting with a positive family history of PC and/or atypical symptoms (rapid progression toward insulinotherapy, instability, and weight loss despite intensive treatment, recurrent infections including mycotic, additional abnormalities in laboratory values, and/or on abdominal sonography), should be investigated with highly sensitive imaging methods, including the preferred use of endosonography, to exclude asymptomatic pancreatic malignancy. There is an urgent need for a biomarker identification that would facilitate the definition of high-risk individuals among newly diagnosed diabetics. This practice could lead to the diagnosis of earlier stages of the disease, allowing the curative surgery and more favorable prognosis. Prospective studies are necessary to verify the potential of high risk groups defined this way and the implications of their screening on morbidity and mortality.

Conflict of Interests

All of the authors have no conflict of interests to declare.

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Review Article

Prostate Carcinogenesis with Diabetes and Androgen-Deprivation-Therapy-Related Diabetes: An Update

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Prostate cancer and the androgen deprivation therapy (ADT) thereof are involved in diabetes in terms of diabetes-associated carcinogenesis and ADT-related metabolic disorder, respectively. The aim of this study is to systematically review relevant literature. About 218,000 men are estimated to be newly diagnosed with prostate cancer every year in the United States. Approximately 10% of them are still found with metastasis, and in addition to them, about 30% of patients with nonmetastatic prostate cancer recently experience ADT. Population-based studies have shown that dissimilar to other malignancies, type 2 diabetes is associated with a lower incidence of prostate cancer, whereas recent large cohort studies have reported the association of diabetes with advanced high-grade prostate cancer. Although the reason for the lower prevalence of prostate cancer among diabetic men remains unknown, the lower serum testosterone and PSA levels in them can account for the increased risk of advanced disease at diagnosis. Meanwhile, insulin resistance already appears in 25–60% of the patients 3 months after the introduction of ADT, and long-term ADT leads to a higher incidence of diabetes (reported hazard ratio of 1.28–1.44). Although the possible relevance of cytokines such as Il-6 and TNF-α to ADT-related diabetes has been suggested, its mechanism is poorly understood.

1. Introduction

Prostate cancer and the hormonal therapy thereof (androgen deprivation therapy, ADT) have been associated with diabetes in terms of diabetes-associated carcinogenesis [1] and ADT-related metabolic disorder [2], respectively. The present paper systematically introduces prostate carcinogenesis with diabetes and ADT-related diabetes/insulin resistance both in epidemiological and etiological approaches.

2. Search Method

PubMed and MEDLINE searches were performed for articles published between January 1991 and November 2011 based on the following key words for diabetes-associated prostate carcinogenesis: prostate cancer AND insulin resistance, hyperglycemia, cancer risk, and diabetes. Literature on ADT-related diabetes was searched using the following keywords: androgen deprivation therapy OR hormone therapy AND diabetes, insulin resistance, hyperglycemia, and metabolic syndrome. Relevant articles on growth hormone (GH)/insulin-like growth factor (IGF)-1 and androgen metabolism were searched with similar strategy. Except for studies concerning statistics, meta-analysis, or reanalysis, review articles were excluded. All full papers based on evidence level 1 and 2 and full papers on level 3 supporting them were downloaded via the library of our institution, provided from other institutions, or purchased, and relevant articles on experimental studies were obtained by similar methods.

3. General Statistics of Prostate Cancer

Prostate cancer is a common malignancy around the world, and in the United States, about 218,000 and 32,000 men are estimated to be newly diagnosed with and to die of prostate cancer every year, respectively [3]. Therapeutic options

for prostate cancer are determined with informed-consent according to the disease-specific risk and patient's conditions such as age and comorbidities. Although the prostate-specific antigen (PSA) test has led to a stage migration with increased low- to intermediate-risk localized disease, about 10% of the patients are still found with metastatic disease at diagnosis [4]. Additionally, 20–35% of the patients are categorized as having locally advanced disease or localized high-risk cancer based on high histopathological grade (Gleason score of 8–10) or high PSA level (serum PSA higher than 20 ng/mL) [5, 6].

4. The Presence of Diabetes and the Incidence of Prostate Cancer

Large cohort studies have shown that diabetes is associated with a higher incidence of many malignancies including lung, gastric, colorectal, liver, and pancreatic cancer [7, 8]. Several molecular mechanisms have been suggested for their association, for example, insulin resistance leading to high cell proliferation by the activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway, elevated leptin/adiponectin linking to impaired anticancer immunity, and upregulated inflammation/tumor necrosis factor-alpha (TNF-α) leading to cancer cell survival; they produce a complex network with many-to-many correspondence [9, 10]. Conversely, the mentioned cohort studies reported a lower prevalence of prostate cancer among men with type 2 diabetes compared with that in men without diabetes. Recently, Turner and colleagues reported that diabetes was associated with a reduced risk of prostate cancer (odds ratio = 0.78; 95% CI: 0.61–0.99) [11], and a study referring to the Swedish national database and nationwide Cancer Registry also showed a lower risk of prostate cancer in a total of 125,126 registered type 2 diabetes men [12]. Most recently, Atchison and associates reported that men with diabetes had a decreased risk of prostate cancer (RR = 0.89, 95% CI = 0.87–0.91) [7]. These results imply a specific relationship between diabetes and prostate cancer; however, it remains unknown why type 2 diabetes is associated with a lower incidence of prostate cancer. In a retrospective study enrolling 3,162 consecutive men who underwent prostate biopsy, Moses and associates showed that, though not significant, those with diabetes had higher odds of histologically more aggressive disease (Gleason score of 7 or higher) than those without diabetes (OR 1.31, 95% CI: 0.98–1.74; $P = 0.07$) [13]. In their study, diabetes also led to an increased risk of overall prostate cancer in the cohort (OR 1.26, 95% CI: 1.01–1.55; $P = 0.04$). It is suggested that the study design and cohort in the study by Moses et al. mainly comprising men with elevated PSA on prostate cancer screening possibly involves different patients' background.

It is known from the results of recent population-based cohort studies that men with type 2 diabetes show lower PSA levels than those without diabetes [14–16]. Considering evidence on reduced PSA levels in diabetic men, is exposure to PSA screening associated with a reduced risk of prostate cancer in men with diabetes? In a longitudinal observational study enrolling 4,511 men with newly diagnosed prostate cancer between 1986 and 2004, Kasper et al. demonstrated the increased risk of prostate cancer in diabetic men after PSA era compared with that in pre-PSA era, although the odds ratio still remained low after PSA era (0.86) [17]. In a recent population-based study conducted in Taiwan, 985,815 study subjects including 104,343 diabetes patients identified in 1997 were followed up between 1998 and 2009; the unadjusted and adjusted risk ratios in diabetes men for incident prostate cancer were 6.97 (5.34–9.10) $P < 0.001$ and 1.56 (1.19–2.04) $P = 0.0013$, respectively [18]. However, the rate of exposure to PSA screening in this population was unclear.

The relevance of lower PSA levels to the reduced risk of prostate cancer in men with diabetes is thus equivocal, but men with diabetes potentially have more advanced disease at diagnosis where their PSA level reaches a certain cut-off/threshold. Correspondingly, two recent large cohort studies reported the association of diabetes with high/poor-risk disease: more advanced clinical stage and higher Gleason sore [1]. In a cohort study, Li et al. reported that men with diabetes had a higher risk of advanced prostate cancer with a multivariate adjusted HR of 1.89 (95% CI: 1.02–3.50) in 230 men with prostate cancer newly identified among 22,458 Japanese men [19]. Although retrospective, several large studies have also reported the relationship between diabetes and high-grade prostate cancer [20, 21].

Accordingly, diabetes is associated with a lower PSA level in the general population and a higher incidence of poor-risk prostate cancer in the screening-based cohort or regional cancer registration. The latter can be explained by the frequent reduced testosterone levels in men with increased insulin resistance or type 2 diabetes [22] and is concordant with previous study results on prostate cancer biology; low testosterone environment *in vivo* is involved in high Gleason score [23–25], advanced disease stages [26], and a poor prognosis [27, 28]. All of these studies have suggested that the adaptation of cancer cells to low-testosterone milieu links to their high viability and malignant potential. Most recently, Botto and associates reported a high incidence of predominant Gleason pattern 4 (histologically high-grade pattern) in men with prostate cancer and low serum testosterone [29]. They performed a prospective study on 452 men who underwent radical prostatectomy; the final study group comprised 431 eligible patients. In surgical specimens, 132 patients (31%) had predominant Gleason pattern 4, and their serum total testosterone level was lower than that in the remaining 299 with predominant lower histological grade (4.00 versus 4.50 ng/mL, $P = 0.001$). In men with predominant Gleason pattern 4, interestingly, the diabetes history was noted more frequently (8.4% versus 2.7%, $P = 0.008$). Accordingly, diabetes is involved in the incidence of high-grade/advanced prostate cancer most probably via the acquisition of more malignant potential under low-testosterone environment.

Meanwhile, the mechanism of lower PSA levels in diabetic men is hard to explain; it is still unclear why diabetes is associated with lower PSA levels. As described elsewhere, serum testosterone levels in men with type 2

diabetes are likely to be lower [22]. Yet, their deference from the normo-gonadotropic testosterone level is about 30% in median. It remains unknown whether the decrease of testosterone levels in such degree has an impact on serum PSA levels. Morgentaler advocated a theory that can account for such contradiction between androgen and PSA levels; there is a limit to the ability of testosterone to stimulate androgenic activities including prostate epithelium proliferation [30]. The Saturation Model explains the observation that prostate epithelium proliferation is testosterone dependent in serum testosterone concentrations at or below the near-castrate level (levels of 95% or more testosterone being deprived) and becomes testosterone-independent above this concentration. Physiologic concentrations of testosterone provide an excess of testosterone and its intracellular prostatic metabolite dihydrotestosterone, which maintains optimal prostatic growth. Reducing testosterone concentration below a critical concentration threshold (the Saturation Point) leads to an intracellular milieu where prostate tissue grows in an androgen-dependent manner [25, 30, 31]. Thus, the mild decrease of testosterone levels in diabetic men does not seemingly explicate their lower serum PSA level.

Another interest is whether a higher insulin level is associated with a higher incidence of prostate cancer. Stocks and colleagues prospectively performed conditional logistic regression analyses on 392 prostate cancer patients and 392 matched controls [32]. In their study, homeostatic model assessment of insulin resistance (HOMA-IR) was lower in the prostate cancer group than in the control group (1.5 ± 0.7 versus 1.6 ± 0.7), and the increasing level of HOMA-IR was associated with the decrease in risk of prostate cancer (Odds ratio = 0.60, 95% CI, 0.38–0.94, $P = 0.03$). In another case-control study by Chen et al. with 174 men in each of the case and control groups, insulin levels had no impact on the risk of incident prostate cancer [33]. In contrast, a recent cohort study with 9-year observation by Hammarsten and associates showed that the prediagnostic insulin level was higher in men with than without incident prostate cancer (fasting serum insulin 12.0 versus 9.0 mU/l, $P = 0.023$), although the study included a small number of prostate cancer patients ($n = 44$) and hazard ratio for the insulin level was unclear [34]. These varied results may possibly depend on study designs and length of the observation period. In a recent case-cohort study on a large registered cohort, Albanes et al. reported that increased insulin levels were associated with increased risks of prostate cancer (OR = 1.50–2.55 among compared insulin quartiles, $P = 0.02$) [35]. Another previous population-based study reported similar results [36].

In etiological approaches, the regulation and metabolism of insulin and IGF-1 are correlated, sharing homologous molecular structures [37], while many studies have shown the impact of high circulating IGF-1 levels on prostate carcinogenesis [38, 39]. This was also established experimentally before PSA era [40]. However, a recent large prospective study as well as previous studies has concluded the absence of correlation between the plasma IGF-1 level and insulin resistance [32]. In genetics, some reported no association between type 2 diabetes risk variants and prostate cancer risk [41], whereas some suggested a possible [42] or inverse [43] association between them.

Most recently, an experimental study reported intracellular *de novo* steroidogenesis promoted by insulin in prostate cancer; Lubik et al. showed that transcription of androgen-metabolic enzymes such as CYP17A1 and 5-α-reductase were upregulated by insulin in a dose-dependent manner in prostate cancer cells LNCaP and 22RV1, which express androgen receptor [44]. In their study, the protein level of CYP17A1 in LNCaP also increased significantly with insulin, and the intracellular level of dehydroepiandrosterone and testosterone increased 18-fold and 60-fold by insulin, respectively, ($P < 0.05$ in both) with PSA secretion increased significantly. These results suggest that insulin may directly promote proliferation of prostate cancer cells. However, these observations are based on an experimental model for castration-resistant prostate cancer, and studies to examine the effect of insulin on prostate tumorigenesis during its early phase or in hormone-naïve cancer are needed.

Thus, the relationship among insulin resistance, testosterone milieu, PSA level, incidence of prostate cancer, and its malignant potential in men with diabetes has not been fully elucidated, and remains a matter of concern for the regulation of prostate carcinogenesis as well as advances in management of prostate cancer in the general population. Further studies are required in both experimental and clinical approaches.

5. Practice of ADT

Both benign and malignant prostatic epithelial cells are well known to receive proliferative stimuli from androgens and to have androgen-dependent bioactivities, and ADT has been the therapeutic mainstay for men with metastasis or recurrent disease following definitive local therapy, although the treatment effect is palliative in most of the former [45]. ADT is performed with surgical castration or injection of gonadotropin-releasing hormone (GnRH) analogues with or without peroral antiandrogens. In 90s, the use of ADT rapidly increased from a small percent to 30% in the United States [46], and ADT has recently been used in about 30% of patients with localized or locally advanced prostate cancer, mainly combined with radiotherapy for intermediate- to high-risk disease [47]. It is estimated that more than 600,000 men receive ADT and that one in two prostate cancer patients experiences ADT in some treatment setting in the United States [48], whereas annual claims for GnRH analogues decreased by 25.1% and 16.8% from 2004 to 2007 in the Medicare and the Veterans Health Administration populations, respectively, most probably due to prevailing intermittent ADT and expectant management policy in increasing awareness about ADT-related adverse effects [49].

6. ADT-Related Insulin Resistance and Diabetes

It is estimated that the 5-year disease-specific survival for men with prostate cancer reaches 98% [3, 6]. In particular,

men with localized prostate cancer almost exclusively die of other causes, and causes of death in them are similar to those of the general male population [50]. Therefore, ADT-related toxicity and the management thereof are critical in clinical practice.

As discussed elsewhere, reduced testosterone levels are associated with insulin resistance and type 2 diabetes in the general population [22]. Insulin resistance appears early during ADT; some previous prospective studies showed that increased fasting insulin levels already emerge in 26–63% of the patients 3 months after the inception of ADT [51, 52]. Hyperinsulinemia during the early period of ADT possibly counteracts against the development of diabetes. Yet, long-term ADT leads to a higher incidence of diabetes as shown in following large population-based studies, although there has been no prospective longitudinal study with a long observation period.

Keating et al. used Surveillance, Epidemiology, and End Results (SEER) Medicare data; the study cohort comprised 73,196 men with localized prostate cancer [53]. Among the 64,721 men without prevalent diabetes, 10.9% developed diabetes, and its adjusted hazard ratio was 1.44 (95% CI: 1.34 to 1.55, $P < 0.001$) in men treated with GnRH agonists. The same authors most recently performed another large population-based study and reported an increased risk of incident diabetes in men undergoing ADT with GnRH agonists (adjusted hazard ratio: 1.28, 95% CI: 1.19 to 1.38, $P < 0.001$) [2]. A Canadian population-based study also showed an increased incidence of diabetes in men treated with GnRH agonists (HR: 1.16, 95% CI: 1.11–1.21, $P < 0.001$) [54]. Another large study, though retrospectively, reported that 8.94% of men who were treated with ADT ($n = 1{,}231$) were diagnosed with diabetes 12 months after ADT, while 6.99% of those without ADT ($n = 7{,}250$) ($P = 0.02$) [55]. Thus, this evidence strongly supports the demand of large well-designed studies that longitudinally analyze the incidence of ADT-related metabolic disorders with long-term followup. Moreover, the pretreatment evaluation and posttreatment followup for diabetes and the relevant conditions are possibly important to improve overall survival in men receiving ADT. However, there has been no interventional study to determine appropriate/efficient screening methods and follow-up interval.

On the other hand, a few trials examined the effect of exercise, diet, and supportive agents/supplements in men during ADT. Nobes et al. reported the efficacy of a low glycemic index diet, exercise program, and metformin (850 mg daily to 850 mg twice daily) in men treated with GnRH agonist (6-month ADT) in a prospective randomized study [56]. The intervention arm ($n = 20$) had a reduction in abdominal girth ($P = 0.05$), weight ($P < 0.001$), and body mass index ($P < 0.001$) compared to controls ($n = 20$). Although the study was designed in a small pilot volume, changes in biochemical markers of insulin resistance did not differ between the two arms during the study. Lebret and colleagues examined the utility of an educational tool-kit consisting of information brochure concerning adverse effects of ADT, practical guidance on lifestyle, recipe booklet for ADT-adapted diet, and lifestyle diary to record and evaluate the life style and body measurement [57]. They recruited more than 500 men with prostate cancer receiving ADT, but the aim of the study was to test a tool-kit designed to improve well-being in patients with prostate cancer, and relevant studies on its impact on metabolic disorder during ADT are warranted.

The etiology of ADT-related diabetes is poorly understood. As mentioned above, increased fasting insulin levels are observed early after the initiation of ADT, suggesting possible primary responses to altered hormonal milieu. Additionally, recent studies showed that 6-month ADT with combined GnRH agonist and antiandrogens is associated with an about 10% increase of serum IGF-1 [58–61]. Although evidence supporting that low testosterone environment directly brings about the increased insulin level is absent, several previous studies have suggested associations among diabetes, cytokines, and sex steroid levels. Proinflammatory cytokines such as interleukin-6 (IL-6), and TNF-α secreted by macrophages and monocytes in response to infection play a critical role in immunity. Type 2 diabetes has been involved in innate immune system disorder with chronic low-grade inflammation [62, 63], and many studies have shown that serum/plasma levels of inflammatory markers represented by TNF-α and IL-6 in patients with elevated fasting blood glucose are independent values predictive of development of diabetes, thereby adipose tissue being the major source of these cytokines [64, 65]. Relevance of elevated serum IL-6, and TNF-α levels to insulin resistance and diabetes has been shown accordingly.

Besides, sex steroids such as 17beta-estradiol (estradiol) and testosterone have been suggested to play a role in modulating inflammation, although relevant studies are limited. A previous study showed that estradiol withdrawal brought about greater expressions of proinflammatory cytokines represented by IL-6 and TNF-α in human monocyte-derived macrophages of premenopausal women [66]. Concerning androgens, an *in vitro* study showed inhibition of IL-6 mRNA transcription and TNF release by dihydrotestosterone [67]. Some clinical trials have shown the influence of testosterone administration on cytokines or inflammation. In the double-blinded placebo-controlled crossover study on 20 hypogonadal type 2 diabetic men by Kapoor et al, although testosterone treatment reduced leptin (-7141.9 ± 1461.8 pg/mL; $P = 0.0001$) and adiponectin levels (-2075.8 ± 852.3 ng/mL; $P = 0.02$), its effect on the TNF-α, IL-6 or CRP level was not significant [68]; the small study volume may possibly lead to a negative result on cytokines.

Most recently, Kalinchenko and associates studied the effect of testosterone replacement on diabetic and inflammatory markers in 184 men with metabolic syndrome and hypogonadism in a randomized, placebo-controlled, double-blinded setting [69]. In the testosterone-treated group, plasma insulin and HOMA-IR decreased compared with those in the placebo-treated group ($P = 0.07$ and 0.04, resp.). Thereby, TNF-α and CRP of the testosterone-treated group declined 30 weeks after treatment compared to those at baseline (19 mg/dL versus 29 mg/L, $P < 0.001$ compared to control and 2.4 ng/l versus 3.5 ng/l, $P = 0.03$ compared to control, resp.); however, IL-6 levels were equivalent

between before and after treatment (1.1 ng/l versus 1.1 ng/l). Additionally, subcutaneous abdominal fat has been shown to be an important index reflecting insulin resistance and relevant inflammation. A recent study focused on increased HOMA-IR (2.50 ± 1.12 to 2.79 ± 1.31, $P < 0.05$) and subcutaneous abdominal fat area 240.7 ± 107.5 to $271.3 \pm 92.8\,cm^2$, $P < 0.01$) [70], while abdominal fat mass has been associated with insulin resistance and the innate immune activation [71].

Regarding the effect of ADT on circulating proinflammatory cytokines, the relevant study has barely been presented. A recent study prospectively examined the relationship between these cytokines and sex steroid levels in the serum in 72 men with localized prostate cancer, who received ADT with GnRH agonists [72]. The authors reported an altered association of interleukin-6 with sex steroids during ADT as follows: before ADT, similar to the previous reports, serum interleukin-6 levels were inversely correlated with serum total-testosterone (Spearman's rank correlation coefficient rs = −0.305, $P = 0.009$) and dihydrotestosterone (rs = −0.308, $P = 0.006$) concentrations, but not correlated with adrenal androgen or estradiol levels. After ADT, in contrast to the pretreatment relationship, interleukin-6 levels were positively correlated with total-testosterone concentrations (rs = 0.343, $P = 0.003$), and were positively correlated also with levels of androstenedione (rs = 0.351, $P = 0.002$) and estradiol (rs = 0.335, $P = 0.004$), suggesting a coordinated regulation emerging between proinflammatory cytokines and sex steroids during ADT. Although the study focusing on ADT-related body composition change concluded the unchanged IL-6 level despite increased %body fat, the alteration of hormonal milieu produced by ADT can theoretically have an influence on the association of proinflammatory cytokines with metabolic activities including insulin sensitivity. However, it remains unclear whether such altered association between sex steroids and proinflammatory cytokines is the primary action with insulin resistance or secondary reaction to reduced testosterone levels, and further studies are warranted to elucidate the mechanism of ADT-related diabetes and to overcome this important adverse effect brought about by ADT.

7. Conclusion

Diabetes is associated with a lower PSA level in the general population and a higher incidence of advanced prostate cancer in the prostate cancer registration-based cohort. Although the mechanism of the former association is unknown, the latter can be explained by reduced testosterone levels in men with increased insulin resistance or type 2 diabetes. Insulin resistance is frequently observed early after the introduction of ADT, and long-term ADT links to the increased risk of development of diabetes. However, the mechanism of ADT-related diabetes remains unclear; a regulatory relationship between proinflammatory cytokines and sex steroids is possibly involved in ADT-related diabetes.

Abbreviations

PSA: Prostate-specific antigen
ADT: Androgen deprivation therapy
GnRH: Gonadotropin-releasing hormone
HOMA-IR: Homeostatic model assessment of insulin resistance.

Conflict of Interests

The author declares no conflict of interests.

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Research Article

Combined Transfection of the Three Transcriptional Factors, PDX-1, NeuroD1, and MafA, Causes Differentiation of Bone Marrow Mesenchymal Stem Cells into Insulin-Producing Cells

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Aims. The goal of cell transcription for treatment of diabetes is to generate surrogate β-cells from an appropriate cell line. However, the induced replacement cells have showed less physiological function in producing insulin compared with normal β-cells. *Methods*. Here, we report a procedure for induction of insulin-producing cells (IPCs) from bone marrow murine mesenchymal stem cells (BM-mMSCs). These BM-mMSCs have the potential to differentiate into insulin-producing cells when a combination of PDX-1 (pancreatic and duodenal homeobox-1), NeuroD1 (neurogenic differentiation-1), and MafA (V-maf musculoaponeurotic fibrosarcoma oncogene homolog A) genes are transfected into them and expressed in these cells. *Results*. Insulin biosynthesis and secretion were induced in mMSCs into which these three genes have been transfected and expressed. The amount of induced insulin in the mMSCs which have been transfected with the three genes together is significantly higher than in those mMSCs that were only transfected with one or two of these three genes. Transplantation of the transfected cells into mice with streptozotocin-induced diabetes results in insulin expression and the reversal of the glucose challenge. *Conclusions*. These findings suggest major implications for cell replacement strategies in generation of surrogate β-cells for the treatment of diabetes.

1. Introduction

Type 1 diabetes is characterised by absolute insulin deficiency caused by T-cell-mediated destruction of pancreatic β-cells. β-Cell replacement is a promising approach for treatment of type 1 diabetes. Islet cell replacement has been considered as the potential cure for diabetes over the past thirty years. However, this treatment is limited by a shortage of pancreas donors and immune rejection against islets. Recently, the methods of obtaining insulin-producing surrogate β-Cells from non-β-cells through induction or genetic engineering have been investigated, which supports a new sight in Type 1 diabetes treatment [1–4].

Transcription factors control biological processes such as differentiation, proliferation, and apoptosis. They bind to the specific sequence within the region of the promoter or enhancer and activate specialized genes' expression. It has been reported that a number of transcription factors were involved in pancreas β-cells' development and function maintaining [5]. It has been reported that PDX-1, NeuroD1 and MafA directly bind to the insulin gene promoter and promote transcription of insulin mRNA and maintenance of β-cell function during pancreatic β-cell differentiation. Further studies have also shown that the transcription of these three genes and their resultant three protein products are crucial for glucose regulation of insulin production [6]. But whether synergistic effect may induce higher insulin expression and promote non-β-cells further differentiated into β-cells in vivo or in vitro is still needed to be further studied.

Recently, MSCs were chosen as target cells for transplantation, because of their ability to differentiate into multiple cell types [7, 8], their ability to elude detection by the host's

immune system [9], and the relative ease of expanding these cells in cell culture [9]. In our study, a combination of these three transcription factors, which all play a crucial role in glucose induction of insulin gene transcription and pancreatic β-cell function, were delivered into mMSCs on adenoviral vectors. After infection, the cells were cultured in defined conditions with epidermal growth factor (EGF) to promote transdifferentiation and were found to have an active endogenous insulin gene.

There are some findings demonstrating the feasibility of inducing a functional alteration in cultured MSCs by expression of a single master pancreatic regulator gene [10, 11]. In the previous study, we have successfully generated IPCs from bone marrow MSCs by introduction of a human insulin gene [12]. After the transfected cells were injected into the liver of mice with diabetes, we found that the hyperglycemia in mice with diabetes could be reversed effectively [13]. However, the cells for transplantation showed weak glucose responsiveness and immaturity. There remains a sizable gap between induced cells and normal islet β-cells. The recent work on induced pluripotent stem cells (iPS cells) and induced neuron formation suggests that a specific combination of multiple transcription factors instead of a single one might be sufficient to directly reprogram adult cells [14, 15]. A number of transcription factors play important roles in the processes of β-cell differentiation and to some extent other genes are responsible for maintaining β-cell function. Here, in the present study, the combination of PDX-1, NeuroD1, and MafA markedly induces insulin biosynthesis and secretion in mMSCs and thereby this is a novel approach to induce insulin-producing surrogate β-cells efficiently for transplantation.

2. Materials and Methods

2.1. Construction of Recombinant Adenovirus Vectors Harboring Target Gene. The genes of mouse transcription factors PDX-1, NeuroD1, and MafA (gene ID: 18609, 18012, 378435) were obtained by total gene synthesis and gene sequencing to validate that the synthesis was correct. The encoding sequences of PDX-1, NeuroD1, and MafA were amplified and ligated with an internal ribosome entry site sequence-green fluorescent protein (IRES-GFP) by PCR, then cloned into a shuttle vector pDONR221 by BP clonase II enzyme mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The corrcet recombinant plasmids were then cloned into the pAD/CMV/V5-DEST adenoviral vectors (Invitrogen, Carlsbad, CA, USA) by LR clonase II enzyme mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Electrophoretic analysis and DNA sequencing were performed to identify the recombinant vectors.

2.2. Adenovirus Production. After the cells were counted, the packaging cell line 293A in logarithmic growth phase was incubated in a 6-well culture plate at 37°C, 5% CO_2 the day before transfection. The sequences of the recombinant adenovirus vectors pAd-Mouse PDX-1-IRES-GFP, pAd-Mouse NeuroD1-IRES-GFP and pAd-Mouse MafA-IRES-GFP were confirmed by gene sequencing and linearized with Pac I and then transfected into the adenovirus packaging cell line 293A using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). 48 hours after transfection, cells were detached and transferred to a petri dish. Fresh nutrient medium was added every two or three days. The supernants were collected from 293A cells when most of the cells showed significant cytopathic effect (CPE). Primary adenoviruses were harvested after 3 times of freeze-thawing of the supernants. The primary adenoviruses were used to infect 293A cells in 10 cm petri dishes to make adenoviruses concentrated. Finally the concentrated adenoviruses were stored at −80°C. The control adenovirus expressing green fluorescent protein (Ad-GFP) was prepared as the above-mentioned method. The titer of the adenovirus was determined by an immune method, as follows: the HEK-293 cells that had been infected with adenovirus in different concentrations were reacted with rabbit antiadenovirus polyclonal antibody (1 : 1000) for 1 hour and were then incubated for additional 1 hour with horseradish peroxidase labeled anti-rabbit antibody. After 3,3′-diaminobenzidine (DAB) staining, the titer of the adenovirus was calculated in terms of the number of brown particles formed in different dilutions.

2.3. Cell Culture. Bone marrow mMSCs from mice were enriched and expanded in vitro by using the whole bone marrow adherence method according to the previous protocol published from our laboratory [13], with slight modifications [16]. Briefly, two-month-old male C57BL/6J mice were sacrificed and soaked in 75% ethanol for 3 minutes. The femurs and tibiae were dissected away from attached muscle and connective tissues, after which the bones were placed in phosphate buffered saline (PBS) on ice. Either of the ends of each tibia and femur was clipped, and then the bone marrow was extruded by inserting a 21-gauge needle into the shaft of the bone and flushing with rinse solution. Rinse solution consisted of PBS (PH 7.2), 2% fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, MD, USA) and 1 mM ethylene diamine tetraacetic acid (EDTA). Bone marrow cells were collected by centrifugalization and resuspended in Dulbecco's-modified eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F12; HyClone, Logan, UT, USA) supplemented with 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (Sigma Chemical Company, St. Louis, MO, USA) and 10 ng/mL human basic fibroblast growth factor (bFGF; ProSpec-Tany TechnoGene, Rehovot, Israel) to promote cell proliferation. Cells were plated in 25 cm^2 culturing flask (Corning Enterprises, Corning, NY, USA) and incubated at 37°C with 5% humidified CO_2. The nonadherent cells were removed after 72 h, and adherent cells were thoroughly washed twice with PBS. As the cells grew to 80% confluence and were treated with 0.25% trypsin-0.02% EDTA (Sigma Chemical Company, St. Louis, MO, USA) for 5 minutes at 37°C in the ratio 1 : 2 at each passage. Flow cytometry was performed for immunophenotype analysis of mMSCs. mMSCs at passage 3 were trypsinized and washed three times with PBS, then the cells were incubated with the following labeled antibodies:

CD14, CD29, CD34, CD44, CD45, and CD105. Labeled cells were analyzed on a FACSort Calibur (BD Biosciences, Franklin Lakes, NJ, USA). To confirm the multipotency character of mMSCs, mMSCs at passage 3 were incubated in osteogenic or adipogenic differentiation medium for 21 days, followed by staining with alkaline phosphatase or oil red, respectively, as described previously [17].

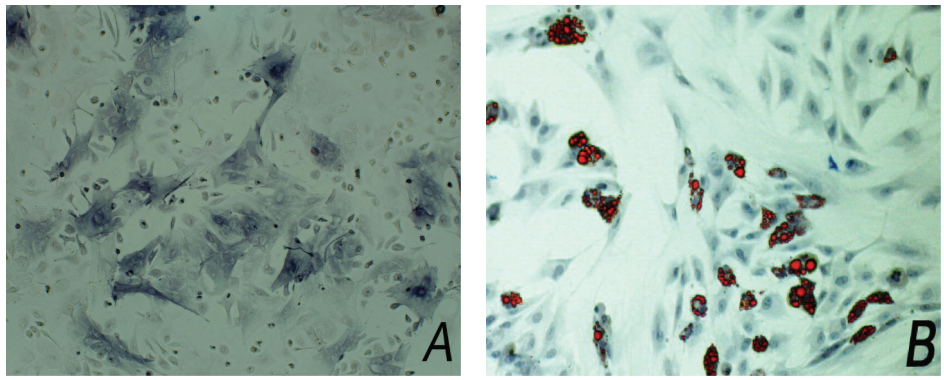
2.4. MOI Determination and Cell Infection. Cells were collected from a highly proliferative mMSC culture at passage 3 and plated into 96 well culture plates at the same density. On the next day cells were infected with freshly harvested Ad-GFP at different multiplicity of infection (MOI) from 5 to 5000 in medium containing 2% FBS and incubated for 24 h. Infection efficiency was determined by fluorescence microscope after a further three days, and toxicity was determined by Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). 10 uL of CCK-8 solution was added into every well according to the instructions on the manufacturers' kit, followed by incubation at 37°C with 5% humidified CO_2 for 2 hours. The absorbance of the infected cells was measured by a microplate reader. The optimal MOI were identified from infection efficiency and toxicity. Ad-Mouse PDX-1-IRES-GFP, Ad-Mouse NeuroD1-IRES-GFP and Ad-Mouse MafA-IRES-GFP were prepared, and then mMSCs were infected with viruses containing the three factors at an optimal MOI. Each of the adenovirus has the same contribution to the optimal MOI, single gene delivery and double infection were also performed. The following day the cells were switched to differentiation medium supplemented with EGF. The infection was repeated in the following days. The cells were infected with Ad-GFP as a control.

2.5. Reverse Transcription Polymerase Chain Reaction. Total cellular RNA was isolated using the MicroElute Total RNA Kit (OMEGA BIO-TEK, GA, USA) according to the manufacturer's instructions. After quantification by spectrophotometry, 2 μg of RNA was used for cDNA synthesis with a RevertAid TM First Strand cDNA Synthesis Kit (Fermentas DNA International, Burlington, Canada). Polymerization reactions were performed using a 20 μL reaction volume containing 1 μL of cDNA, with the oligonucleotide primers being as follows: PDX-1 (330bp), 5′-TGAAATCCACCAAAGCTCACGC-3′ (forward primer) and 5′-CCGAGGTCACCGCACAATCT-3′ (reverse primer); NeuroD1(494bp), 5′-GAGGAACACGAGGCAGACAAG-3′ (forward primer) and 5′-AAGAAAGTCCGAGGGTTGAGC-3′ (reverse primer); MafA (402 bp), 5′-CCATCATCACTCTGCCCACCAT-3′ (forward primer) and 5′-CCCGCCAACTTCTCGTATTTCT-3′ (reverse primer); insulin1 (327 bp), 5′-CTATAAAGCTGGTGGGCATCC-3′ (forward primer) and 5′-AACGCCAAGGTCTGAAGGTC-3′ (reverse primer); insulin2 (368 bp), 5′-AGCCTATCTTCCAGGTTATTGTTTC-3′ (forward primer) and 5′-GGTGGGTCTAGTTGCAGTAGTTCTC-3′ (reverse primer); β-actin (517 bp), 5′-ATATCGCTGCGCTGGTCGTC-3′ (forward primer) and 5′-AGGATGGCGTGAGGGAGAGC-3′ (reverse primer). Amplification conditions included initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 30 sec, at last an extension step of 10 min at 72°C.

2.6. Immunofluorescence Analysis. After being cultured for 21 days, infected cells were seeded on glass slides in a 12-well culture plates and fixed in 4% paraformaldehyde in PBS. Permeabilizing and blocking was performed in 10% fetal calf serum, 3% bovine serum albumin, and 0.2% triton X-100 in PBS. Then the cells were incubated with primary antibody (rabbit anti-mouse insulin polyclonal antibody 1 : 50; Santa Cruz Biotechnology, CA, USA.) overnight at 4°C. For insulin staining, the cells were further incubated for 2 h at room temperature in the dark, with secondary antibody (Cy3 anti-rabbit 1 : 50; Proteintech Group, Chicago, IL USA). After Hoechst staining for additional 15 min, the slides were washed and examined under the microscope. Images were captured using an Olympus phase contrast fluorescent microscope (Olympus Corporation, Tokyo, Japan).

2.7. Insulin Secretion Assay. The infected cells or noninfected cells were preincubated with Krebs-Ringer buffer (KRB) for 1 h, followed by incubation for an additional 1 h in KRB containing 10.0 mM glucose. The buffer was collected and frozen at −80°C until assay. Insulin enzyme-linked immunosorbent assay (ELISA; Cusabio Biotech Co., Wuhan, Hubei, China) was used for the quantitative determination of insulin levels in the collected buffer according to the manufacturer's protocol. All values were determined against a standard curve prepared with mouse insulin.

2.8. Establishment of Diabetes Mellitus Models and Cell Transplantation. To set up models of mice with diabetes, adult C57BL/6J mice were injected intraperitoneally with Streptozotocin (STZ; Sigma Chemical Company, St. Louis Missouri, USA) at a dose of 160 mg/kg. Hyperglycemia had been made by the administration of this dose of STZ within 7 days. Blood glucose reached levels >16.7 mmol/L and kept hyperglycemia for 2 weeks at least. Cells for transplantation were prepared at the same time. Transcription factors PDX-1, NeuroD1, and MafA were delivered into mMSCs 3 days before transplantation. Mice were anesthetized with intraperitoneal injection of sodium pentobarbital at 50 mg/kg, followed by the abdominal incision. About 1-2 $\times$ 10^6 infected cells or non-infected cells suspended in 0.2 ml PBS were transplanted into the liver parenchyma of mice with diabetes. For the glucose tolerance test, mice were injected intraperitoneally with 2.0 g of glucose per kg body weight after overnight fast. Blood glucose levels were monitored at the indicated time points (0–120 min) in samples obtained from the tail vein of mice by using One-Touch II portable blood glucose monitor (Lifescan Inc., Milpitas, CA, USA). The mice were sacrificed two weeks after transplantation. To witness the survival of the IPCs and detect the insulin secretion of triple infected cells in the liver tissues, the livers were removed and fixed in 10% formalin. Forty-eight hours later they were cut into serially sections



(a) (b)

Figure 1: mMSC were induced into osteoblasts and adipocytes in vitro under different differentiation medium. After incubation for 21 days, the differentiated cells were stained with alkaline phosphatase (a) or oil red (b) for their multipotent characteristic.

and analyzed by immunohistochemistry. Negative controls were also set up. mMSCs infected with Ad-GFP or mMSCs without any infection were transplanted into the livers as a control. Terminal deoxynucleotidyl transferase-mediated biotinylated-dUTP nick-end labeling (TUNEL) assay was also performed using the In Situ Cell Death Detection Kit, Fluorescence (Roche Applied Science, Mannheim, Germany) to determine whether the injected cells were apoptotic. The livers were taken out immediately for making frozen tissue sections. Frozen tissue sections were rinsed with PBS and treated with 1% Triton X-100 in PBS for 2 min on ice. Slides were rinsed in PBS and incubated for 60 min at 37°C with 50 μL of TUNEL reaction mixture. After washing with PBS, the slides were analyzed with fluorescence microscopy.

3. Results

3.1. Production of Adenovirus Harboring PDX-1, NeuroD1 or MafA. The sequences of the resultant recombinant adenoviruses, which encode PDX-1, NeuroD1, MafA, and GFP, were confirmed by gene sequencing and restriction endonuclease digestions with Pac I. Pac-I-digested adenoviral vectors were transfected into the 293A cell line to produce a crude adenoviral stock and the adenovirus was amplified by infecting 293 A cells. Various kinds of cells can be infected with this adenovirus.

3.2. Derivation and Characterization of mMSCs In Vitro. The unattached cells from the bone marrow samples were removed through medium changes, and the adherent mMSCs were cultured for propagation. After subsequent passaging, most of the adherent cells exhibited fairly uniformly appearance. Immunophenotypes of the cells at passage 3 were assayed by flow cytometry analysis. The majority of the cells expressed high levels of CD29, CD44, and CD105. Meanwhile, the markers CD14, CD34, and CD45 displayed extremely low expression. mMSCs were incubated in osteogenic and adipogenic differentiation medium to identify the multipotency character, and it showed osteogenic differentiation and adipogenic differentiation after 21 days (Figure 1). These results indicated that the cultured cells were in the undifferentiated state and distinguished from haemopoietic stem cells.

3.3. Optimizing the MOI of mMSCs. The susceptibility of different types of cells to adenovirus is variable and differs significantly among the cell types. To achieve the optimal infection with adenovirus, we chose the optimal MOI to raise the infection efficiency and also to have the least amount of cytotoxicity to the mMSCs simultaneously. The results indicated that the infection efficiency improved constantly with the increasing MOI. When cells were infected with freshly adenovirus at MOI of 100, the infection efficiency was over 80%. When cells were infected with fresh adenovirus at an MOI of more than 2,400, the infection rate was almost 100%. On the other hand, the cell survival became significantly inhibited with increasing MOI values beyond 600. The survival curves showed that the number of living infected cells decreased markedly when the MOI value was greater than 600 (Figure 2). From what had been shown above, we conclude that infection of cells at an MOI of 600 is the optimal choice. The cells were infected with viruses containing the three factors at an MOI of 600. mMSCs were infected with diverse single recombinant adenovirus, respectively, at an MOI of 200. mMSCs were infected with viruses containing any two of these factors at an MOI of 400. The infected cells became round in morphology and gave off strikingly bright green fluorescence 3 days after infection.

3.4. Combination of PDX-1, NeuroD1, and MafA Induces IPCs from mMSCs Significantly In Vitro. To determine whether the endogenous insulin gene started transcription, gene expression profiles of exogenous transcription factors and insulin gene were evaluated by RT-PCR. As illustrated in Figure 3, the insulin gene and the transcription factor genes were expressed in mMSCs, which were infected with the corresponding transcription factors. The effect of combination of the three factors was more profound compared with any other groups. Consequently, the amount of insulin1 and insulin2 mRNA expression in mMSCs with triple infection

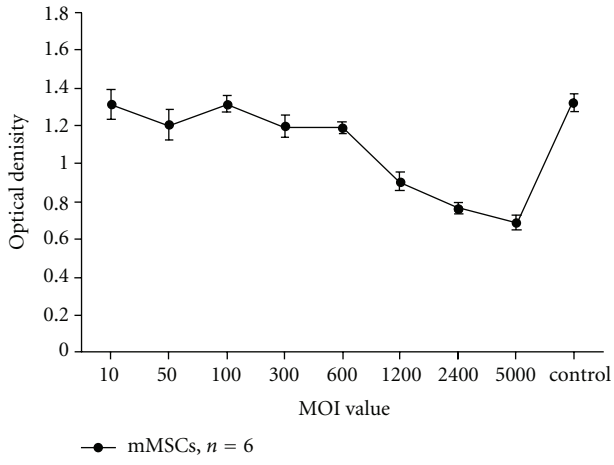


Figure 2: The toxic effect of adenovirus on mMSCs at different multiplicity of infection was determined by CCK-8. 10 μL CCK-8 solution was added into the same amount of mMSCs which were infected with Ad-GFP at different MOI. Optical density of each well varied directly with the survival of the cells. This experiment was repeated six times. Values are mean ± SD.

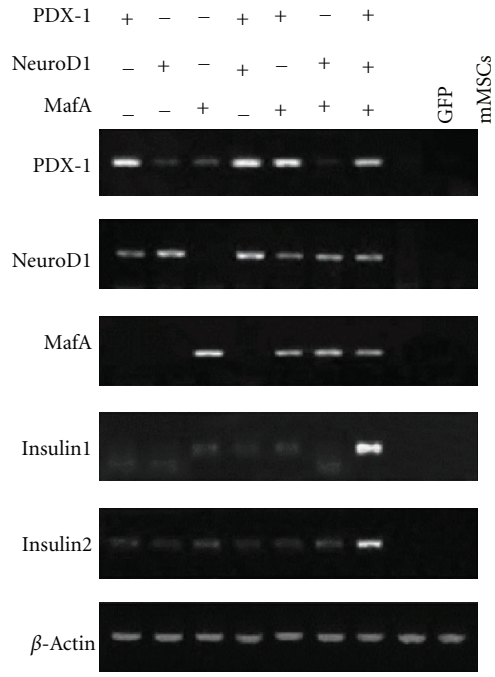


Figure 3: Adenovirus-mediated expression of PDX-1, NeuroD1, and MafA together induced expression of the insulin gene in infected mMSCs. mMSCs were infected with diverse single recombinant adenoviruses, both of the two adenoviruses, a combination of the three adenoviruses, or Ad-GFP. Total RNA from mMSCs was isolated 3 d after infection, and RT-PCR analysis was performed to examine expression of the specified genes. Cultured mMSCs without infection served as the negative control.

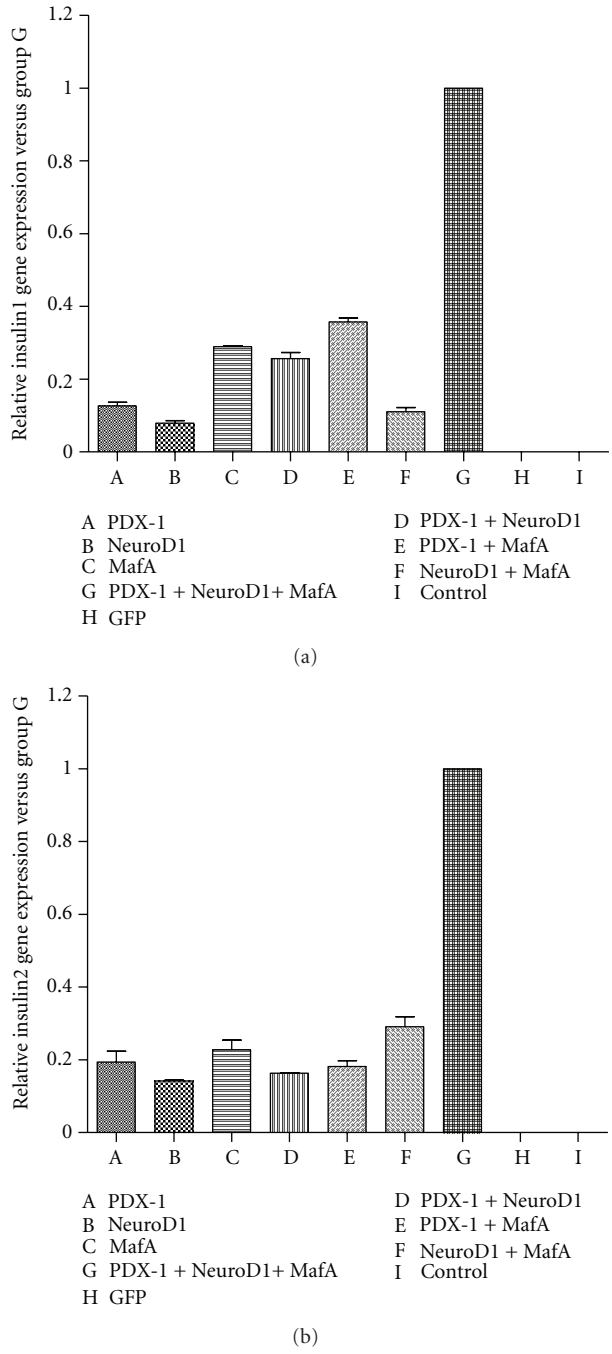
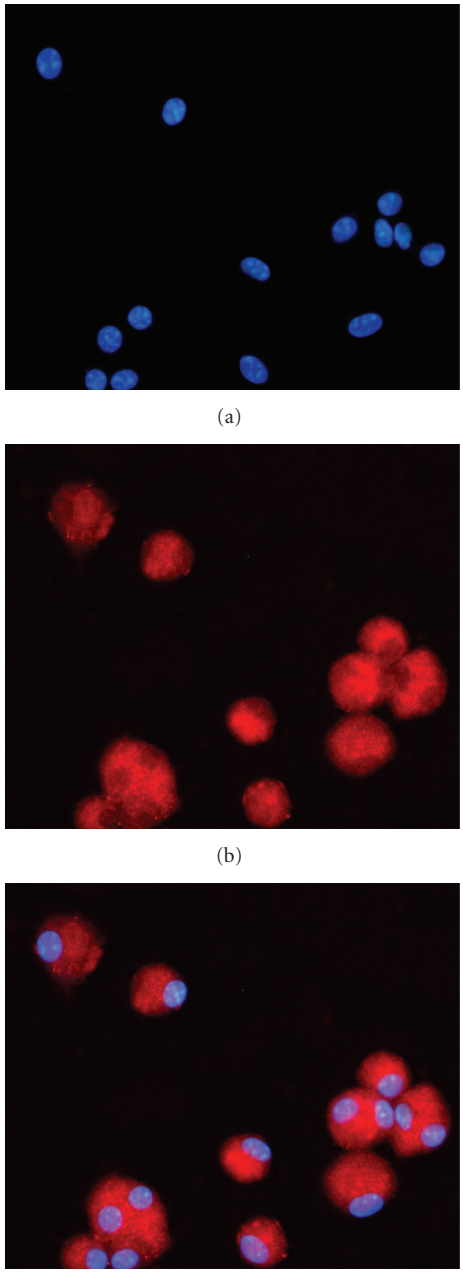


Figure 4: Quantitation of the amount of insulin1 and insulin2 that were produced by the infected mMSCs. The amount of insulin1 (a) and insulin2 (b) mRNA expression in mMSCs with triple infection was significantly larger compared with any other group.

was also much larger than any other groups (Figure 4). In contrast, mMSCs infected with Ad-GFP or null, treated with the same culture condition expressed no detectable level of insulin gene or transcription factor gene. It is worthwhile to note that transcription of PDX-1 and NeuroD1 could be mutual activating, and exogenous MafA could trigger the expression of the endogenous PDX-1 in mMSCs.

After infection, to determine the biosynthesis of insulin and assay the insulin expression at the protein level, the differentiated mMSCs were documented by immunofluorescence analyses. All the cells on the slides were incubated with



(a)

(b)

(c)

Figure 5: Expression of insulin protein in the infected cells. After culturing them for 21 days, all the infected cells were incubated with anti-mouse insulin antibody. Nuclei were stained blue with Hoechst dye (a). Most of the transgenic cells were stained positively for insulin (b). In contrast, the mMSCs infected with Ad-GFP or null were negative for insulin.

anti-mouse insulin and then red fluorescence was clearly visualized in the nucleus and cytoplasm after the triple infection (Figure 5). Nuclei were stained blue with hoechst dye. Red positive reactions were also observed after single factor infection or both of the two factors infection. In contrast, the cultured cells infected with Ad-GFP or null were negative for insulin.

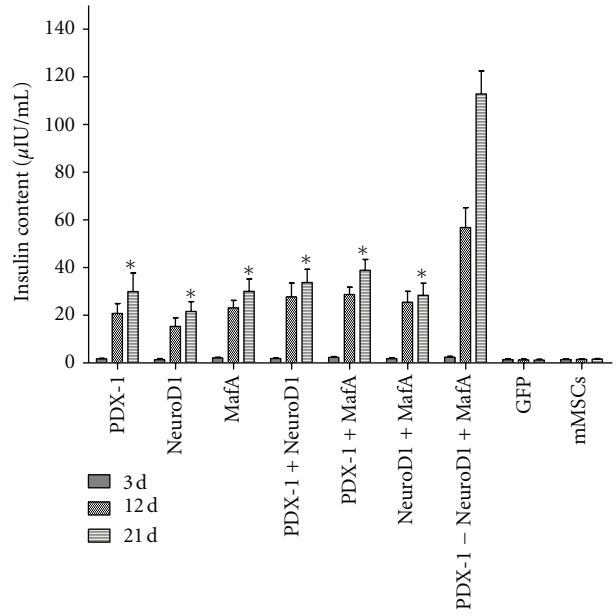
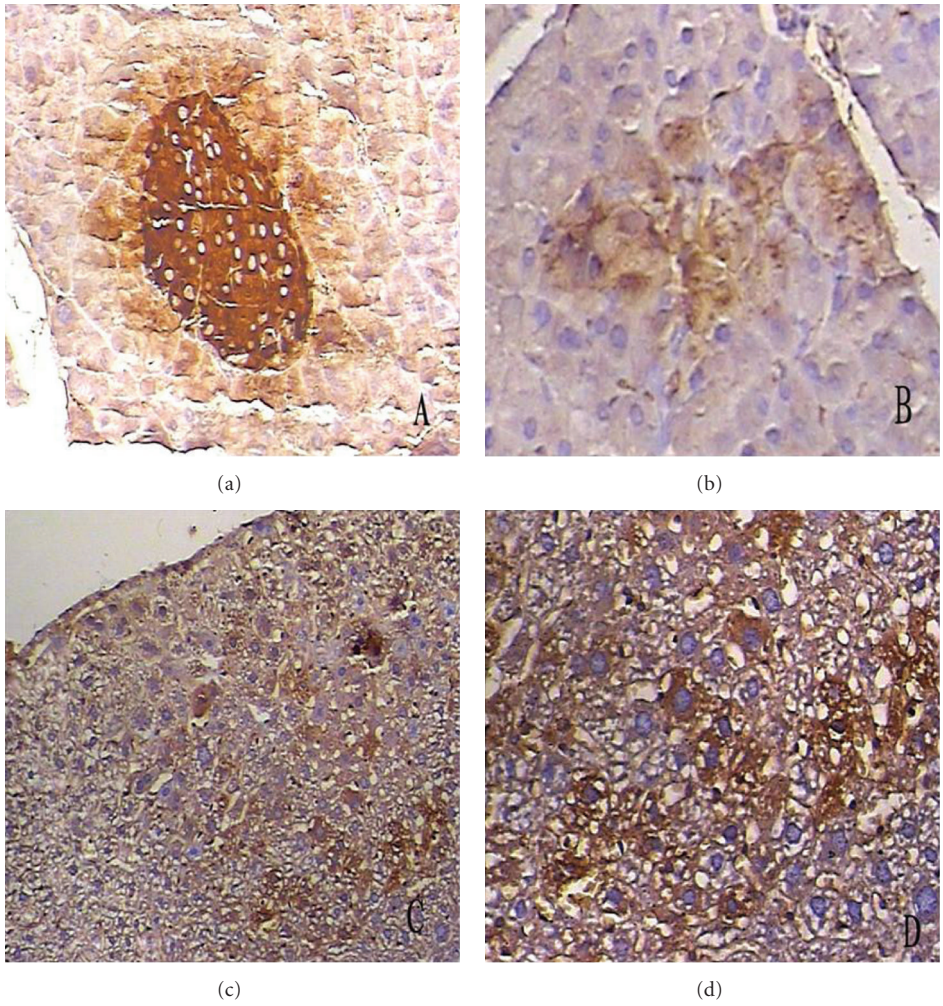


Figure 6: Insulin secretion of the infected cells which were transferred into PDX-1, NeuroD1, MafA, or GFP at different differentiation stages in vitro. The cells were incubated in KRB containing the indicated concentration of glucose. The buffer was then collected for assay of insulin release in each experimental group. One asterisk, $^*P < 0.05$. Data are presented as mean ± SD.

To further determine whether the function of insulin secretion of the differentiated mMSCs, the amount of insulin released by the cells in vitro at indicated concentrations of glucose was measured using a mouse insulin ELISA kit. As illustrated in Figure 6, the results showed that the insulin content of the differentiated mMSCs which were infected with combination of the three transcription factors was significantly higher than that of any other groups ($P < 0.05$). In addition, no insulin release was detected in the buffer added to the cells infected with Ad-GFP or null.

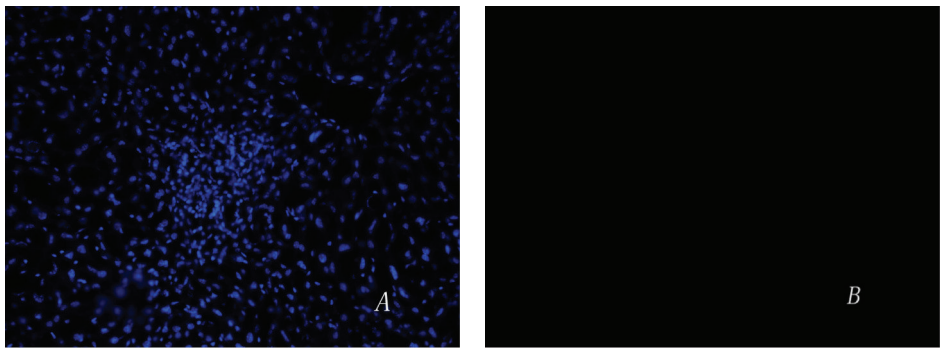
3.5. Function Identification of Induced Insulin-Producing Cells In Vivo. Take a step further to determine whether the induced IPCs give full scope to normal physiological functions of β islet cells, mMSCs expressing a combination of PDX-1, NeuroD1, and MafA were transplanted into the livers of mice with STZ-induced diabetes. mMSCs infected with Ad-GFP or without any infection were transplanted into the livers as a control. First, we examined insulin protein expression and cell apoptosis in the tissue of liver. Insulin content was not detected in the liver of mice treated with mMSCs without infection but was indeed clearly detected after treatment with mMSCs expressed combination of PDX-1, NeuroD1, and MafA (Figure 7). The immunofluorescent stainings of TUNEL were negative in the injected cells (Figure 8), which indicated that they had never experienced double-strand DNA breaks associated with apoptosis. In addition, insulin protein expression was substantially diminished after 1 month and was not detectable after 2 months. Furthermore, to assess the contribution on controlling blood



(a) (b)

(c) (d)

Figure 7: Immunohistochemistry assay for insulin of the survival infected mMSCs in the liver tissues of mice with diabetes. (a) Positive control, anti-mouse insulin staining of mouse pancreatic specimen showing an intense expression of insulin (enlargement ×100). (b) Anti-mouse insulin staining of pancreatic specimen of mice with STZ-induced diabetes showing a markedly decreased expression of insulin (enlargement ×100). (c) Infected mMSCs were injected into the livers of mice with diabetes three days after infection. The positive staining of mouse insulin expression can be clearly observed in the liver. (d) An enlargement of induced IPCs in the liver.



(a) (b)

Figure 8: TUNEL assay was performed to see whether the injected cells were apoptotic. (a) Frozen tissue sections of the livers were stained with hoechst. (b) The immunofluorescent stainings of TUNEL were negative in the transplanted cells.

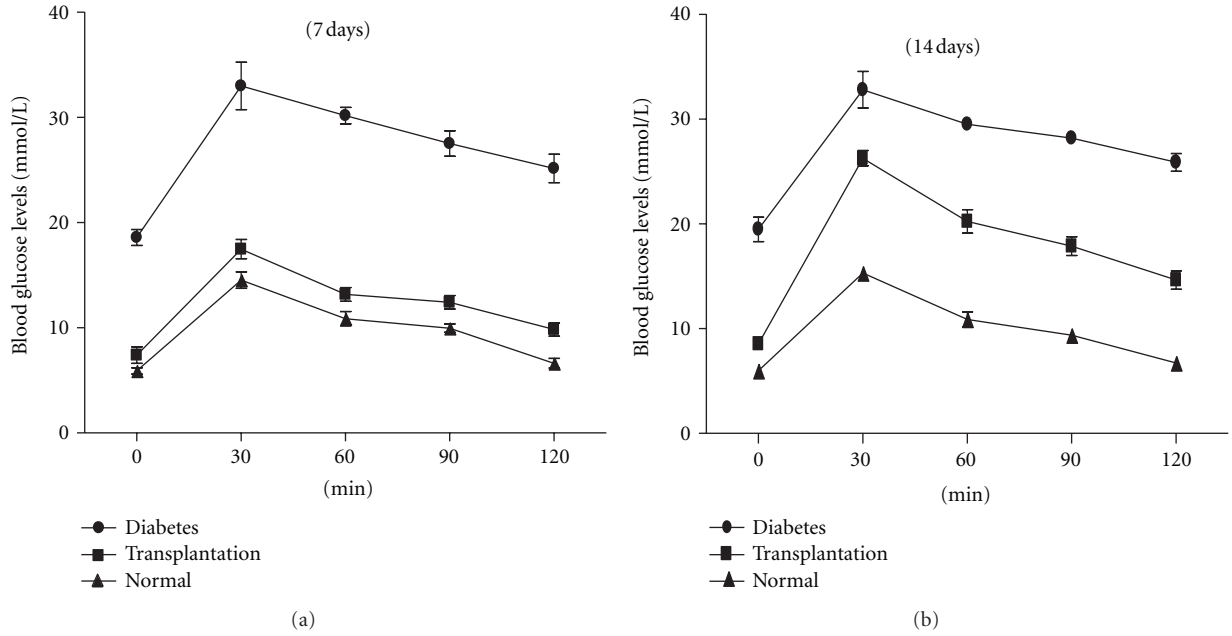


Figure 9: Glucose responses to glucose tolerance test of mice with diabetes after transplantation. The infected cells which expressed combination of PDX-1, NeuroD1, and MafA were transplanted into the livers of mice with STZ-induced diabetes, glucose tolerance test was performed at 7 (a) and 14 days (b) following transplantation, compared with a normal control and diabetes models without any treatment. Data are presented as mean ± SD.

glucose levels of insulin produced by the engrafted cells, a glucose tolerance test was performed 7–14 days after transplantation. As shown in Figure 9, the result revealed that mMSCs expressing a combination of PDX-1, NeuroD1, and MafA were able to respond to the glucose challenge, and their response was almost comparable to that of normal β-islet cells 7 days after transplantation. Notably, the same or better effect was not elicited after another 7 days. This is probably due to the fact that unstable and transient transgene expression in the cells, plus induced cells failed to materialize self-reproduction. There were no differences in blood glucose levels at any time point between mice with STZ-induced diabetes implanted with normal mMSCs and nontransplantation.

4. Discussion

In recent years, cell transplantation has become a research hotspot concerning surgical methods for the treatment of diabetes. In order to obtain surrogate β-cells, the target cells were transdifferentiated, dedifferentiated, or differentiated to surrogate β-cells in the usual by expressing some key transcription factors involved in the pancreas development and β-cell gene expression [18, 19]. In this study we report a procedure for delivery of combination of PDX-1, NeuroD1, and MafA into mMSCs on adenoviral vectors and their differentiation into IPCs in vitro or in vivo. Our results demonstrate that bone marrow mesenchymal stem cells were induced for directional differentiation into IPCs by a combination of just three key transcription factors. Overexpression of PDX-1, NeuroD1, and MafA markedly upregulated the expression of insulin gene and also induced insulin biosynthesis and secretion in mMSCs. The triple infection has a much stronger influence compared with any single or double infection.

The β islet cells are unique in their ability to produce, process, and secrete significant amounts of insulin in a strictly regulated manner in response to continuously varying concentrations of glucose [20]. The development process and function maintenance of β-cells demand networking regulation consisting of several transcription factors.

Previous research has suggested that stable expression of PDX-1 in adult human mesodermal tissues activated expression of all four islet hormones including insulin and reversed hyperglycemia in vivo, but more factors that stimulate cells further toward differentiated normal β-cells were needed [10]. In our study, any single factor and combinations of any two factors were able to induce expression of insulin, but the effect elicited in mMSCs was too weak relative to the particular combination of these three factors. It is apparently not sufficient to drive differentiation of mMSCs a long way toward β-cells or IPCs in the treatment of diabetes. A certain fact to be reckoned with is that all the three transcription factors are bound to the A3, E1, and C1 sites in a 340 bp promoter region upstream of the transcription start site of the insulin gene [21–25]. In contrast or for further research, we developed our experiments in vivo so that induced IPCs would reside in their native environment and might be promoted in their survival and maturation. As the homologous feature between the liver and the pancreas has been displayed in many animal samples [26], transplantation experiments

and in vitro differentiation experiments [27], in addition that the liver is the primary organ where insulin functions, we think the liver tissue is an ideal microenvironment for IPCs to survive and function. Further work will be to explore if additional factors are necessary for the particular combination and mechanism among actions of the factors.

In the experiments of gene detection, genetic transformation of PDX-1 activated the expression of endogeneous NeuroD1 and endogeneous PDX-1 could be activated by exogenous NeuroD1 or MafA. The experimental results indicated that adjustment or interaction may really exist between each transcription factor. However, PDX-1 and MafA, together with endogeneous NeuroD1 were unable to exert as strong an influence on the expression of the insulin gene as delivery of a combination of the three transcription factors. We assume that fine synergism could not be achieved due to the low expression level of induced factors.

Intracellular GFP of the mMSCs was subsequently initiated to expression at 3 days after gene delivery, close together with the factors. However, one week, later, the intensity of the fluorescence decreased with the degradation of partial mitochondrial DNA. Therefore, induced efficiency was significantly inhibited without a repetition of infection. Cell transplantation in liver parenchyma was done to further verify the function of induced IPCs in vivo. Both intraperitoneal injection and high carbohydrate feeding are the methods recommended by researchers for glucose tolerance test. Comparatively, intraperitoneal injection goes in a more accurate way for mice and is also simple to perform. The results of an IPGTT demonstrated the ability of these implanted cells to dispose of a glucose load, and the glucose tolerance was close to normal mice. However, it should be noted that impaired glucose tolerance was found after another 7 days. It may be the case that the implanted induced IPCs failed to proliferate. Strategies that make stable expression of the factors in mMSCs may possibly help to evaluate the long term effect of the treatment.

Bone marrow mesenchymal stem cell has been known for their multiplex differentiation potential and relative ease to obtain. They were able to be modified to develop epigenetic changes, which were controlled by a series of several distinct related genes, and then differentiated into functional β-cells.

In conclusion, our findings demonstrated that genetic manipulation producing infection by a combination of PDX-1 NeuroD1, and MafA and their subsequent expression significantly promoted insulin-producing function of mMSCs. Although substantial work has been done, the effective approach related to generation of surrogate β-cells for the treatment of diabetes is still not obtained. Nevertheless, we will further identify the differentiation of mMSCs expressed combination of the just three factors in vivo and their stable long-term expression for maintaining strict blood glucose levels.

Acknowledgment

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Review Article

Diabetes and Thyroid Cancer Risk: Literature Review

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Diabetic patients have a higher risk of various types of cancer. However, whether diabetes may increase the risk of thyroid cancer has not been extensively studied. This paper reviews and summarizes the current literature studying the relationship between diabetes mellitus and thyroid cancer, and the possible mechanisms linking such an association. Epidemiologic studies showed significant or nonsignificant increases in thyroid cancer risk in diabetic women and nonsignificant increase or no change in thyroid cancer risk in diabetic men. A recent pooled analysis, including 5 prospective studies from the USA, showed that the summary hazard ratio (95% confidence interval) for women was 1.19 (0.84–1.69) and was 0.96 (0.65–1.42) for men. Therefore, the results are controversial and the association between diabetes and thyroid cancer is probably weak. Further studies are necessary to confirm their relationship. Proposed mechanisms for such a possible link between diabetes and thyroid cancer include elevated levels of thyroid-stimulating hormone, insulin, glucose and triglycerides, insulin resistance, obesity, vitamin D deficiency, and antidiabetic medications such as insulin or sulfonylureas.

1. Introduction

During the past several decades, the prevalence of diabetes has markedly increased [1–3]. Diabetes is associated with increased risk of various kinds of cancer, such as colon cancer, pancreatic cancer, breast cancer, bladder cancer, prostate cancer, and non-Hodgkin's lymphoma [4–8]. Meanwhile, the incidence of thyroid cancer is rising at a rate that is among the fastest of all malignancies [9]. According to a survey in the United States, the incidence of thyroid cancer increased by 2.4-fold from 1973 to 2002, and 87% of the increase consisted of cancers measuring 2 cm or smaller, but the mortality from thyroid cancer was stable [10]. It is believed that the major cause of this increase in incidence is the enhanced detection of early-stage tumors by the use of thyroid ultrasound and ultrasound-guided fine needle aspiration cytology examination. However, this cannot explain the increased prevalence preceding the widespread use of ultrasound [11]. It also cannot explain the increased incidence of large (>5 cm) papillary thyroid cancer [12]. Therefore, there may be some other contributing factors of the increased incidence of thyroid cancer. According to epidemiologic studies, exposure to ionizing radiation is the only clearly established risk factor [13]. Benign thyroid conditions and inadequate or excess iodine intake are the possible risk factors of thyroid cancer [13]. None of them can explain the increased thyroid cancer incidence. Statistic analysis showed that diabetes, obesity, and metabolic syndrome were potential risk factors of cancer development [14–16]. It is not clear whether diabetes plays a role in thyroid cancer risk. In this paper, we review the literature reporting the relationship between diabetes mellitus and thyroid cancer (summarized in Table 1) and the proposed mechanisms linking such an association (depicted in Figure 1).

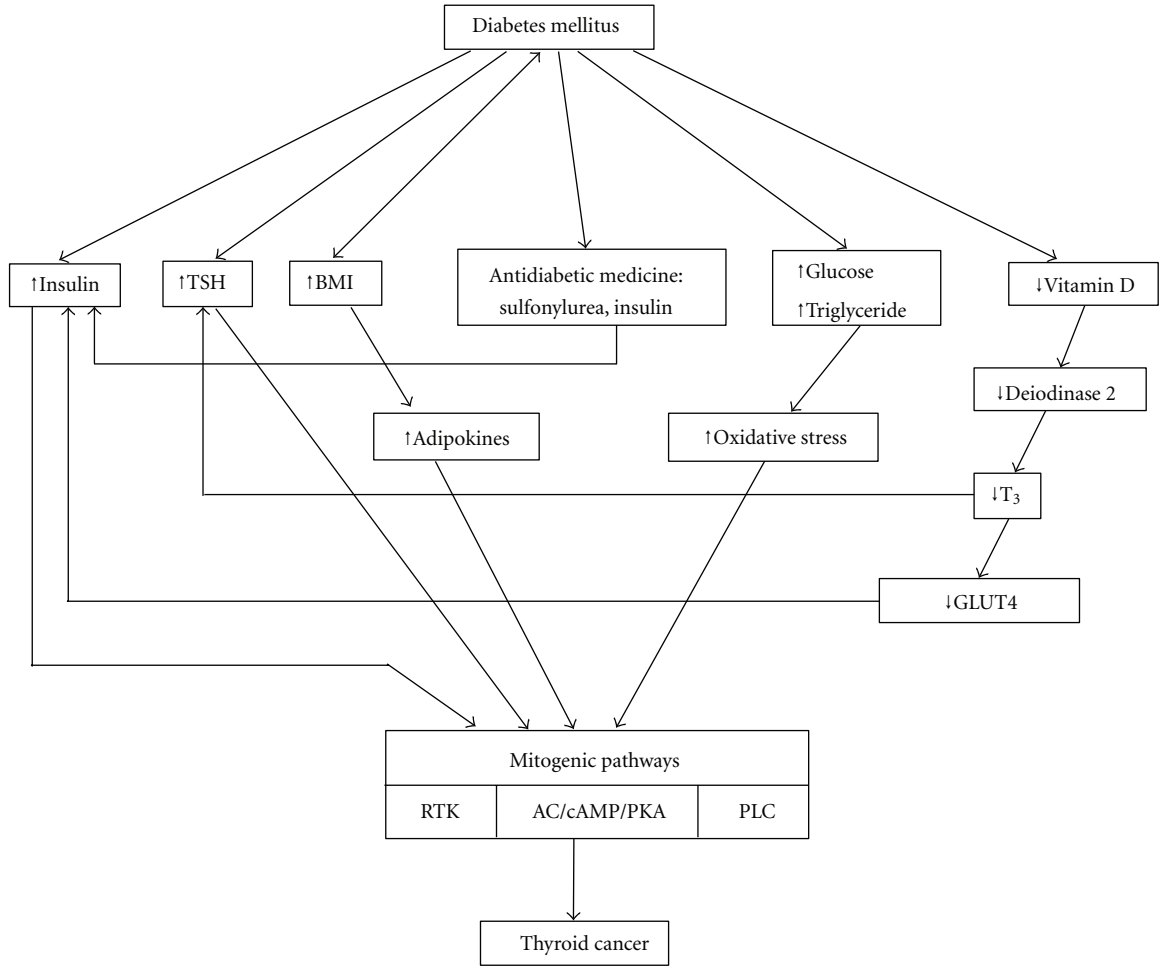
2. Epidemiologic Findings

The prevalence of thyroid disorders among diabetics (10.8%) is higher than that in the general population (6.6%) [24]. Several studies disclosed the relationship between diabetes and thyroid cancer (Table 1) [13, 17, 18, 20–23]. Study

Table 1: Summary of available studies evaluating the relationships between diabetes and thyroid cancer.

Study no. [reference]	Year author	Country	Name of study	Study design	Number of cases/ Follow-up duration	Estimated risk
1 [17]	1991 Adami et al.	Sweden	Cancer risk in patients with diabetes mellitus	Population-based cohort study	51,008 patients. Cohort established by identifying diabetic patients during 1965–1983. Complete followup through 1984	Women: RR = 1 (95% CI: 0.6–1.8) Men: RR = 1.3 (95% CI: 0.5–2.8)
2 [18]	1997 Wideroff et al.	Denmark	Cancer incidence in a population-based cohort of patients hospitalized with diabetes mellitus in Denmark	Prospective cohort study	109,581 diabetics. Cohort established by identifying diabetic patients during 1977–1989. Cohort exit date: date of death or 1993	Women: SIR = 1.3 (95% CI: 0.6–2.3) Men: SIR = 1.2 (95% CI: 0.7–1.8)
3 [19]	2006 Inoue et al.	Japan	The Japan Public Health Center-based Prospective Study	Prospective cohort study	46,548 women, 51,223 men Followed from 1990 through 2003	Women: HR = 1.11 (95% CI: 0.35–3.5) Men: NA
4 [20]	2007 Kuriki et al.	Japan	Hospital based Epidemiologic Research Program at Aichi Cancer Center, Japan	Case-control study	11,672 incident cancer cases (5341 men, 6331 women) 47,768 cancer-free controls (14,199 men, 33,569 women)	Women: OR = 0.67 (95% CI: 0.21–2.10) Men: OR = 1.07 (95% CI: 0.33–3.48)
5 [13]	2010 Meinhold et al.	USA	The US Radiologic Technologists Study	Prospective cohort study	69,506 women, 21,207 men Followed from 1983 through 2006	Women: HR = 1.37 (95% CI: 0.49–3.77) Men: NA
6 [21]	2010 Chodick et al.	Israel	Diabetes and risk of incident cancer: a large population-based cohort study in Israel	Retrospective cohort study	16,721 DM, 83,874 non-DM Mean follow-up time: 8 years	Women: HR = 1.61 (95% CI: 0.96–2.69) Men: HR = 0.72 (95% CI: 0.25–2.04)
7 [22]	2011 Aschebrook-Kilfoy et al.	USA	The NIH-AARP Diet and Health Study	Prospective cohort study	200,556 women, 295,992 men Mean follow-up time: 10 years	Women: HR = 1.54 (95% CI: 1.08–2.20) Men: HR = 1.11 (95% CI: 0.74–1.66)
8 [23]	2012 Kitahara et al.	USA	Physical activity, diabetes, and thyroid cancer risk: a pooled analysis of five prospective studies	Pooled analysis of five prospective studies, including NIHAARP Diet and Health Study (NIH-AARP), Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO), Breast Cancer Detection and Demonstration Project (BCDDP), Agricultural Health Study (AHS), and US Radiologic Technologists Study (USRT)	312,149 women, 362,342 men Median follow-up time: 10.5 years	Women: HR = 1.19 (95% CI: 0.84–1.69) Men: HR = 0.96 (95% CI: 0.65–1.42)

Studies no. 5 and 7 were included in the pooled analysis of study 8. CI: confidence interval, HR: Hazard ratio, OR: odds ratio, RR: relative risk, SIR: site-specific standardized incidence ratio, NA: not available.



AC/cAMP/PKA: the hormone receptor adenylate cyclase cAMP protein kinase A system

BMI: Body mass index

GLUT4: Glucose transporter 4

PLC: The hormone receptor phospholipase C cascade pathway

RTK: The hormone receptor tyrosine protein kinase pathways

T_3: Triiodothyronine

TSH: Thyroid-stimulating hormone

Figure 1: Pathophysiology proposed to link diabetes and thyroid cancer together. Diabetes mellitus may affect mitogenic pathway of the follicular cells through several mechanisms. Increased insulin amount stimulates follicular cells because of its structural similarity to insulin-like growth factor. Increased TSH stimulated AC/cAMP/PKA pathway. Increased body mass index will increase adipokines and subsequently stimulate mitogenic pathways. Antidiabetic medicines of sulfonylurea and insulin contribute to the elevated insulin level. Hyperglycemia and hypertriglycemia increase oxidative stress and stimulate mitogenic pathway. Vitamin D deficiency decreases deiodinase 2, T_3 and GLUT4 transcription, which subsequently increase TSH and insulin levels and activate mitogenic pathways.

number 8 [23] in Table 1 is a pooled analysis including 5 prospective studies from the USA, which included studies numbered 5 [13] and 7 [22]. Significant or nonsignificant increases in thyroid cancer risk were observed in diabetic women [13, 18, 19]. Nonsignificant increases or no change in thyroid cancer risk were observed in diabetic men [13, 17, 18, 20]. The results are controversial, and the link between diabetes and thyroid cancer is probably weak.

3. Hypotheses Proposed to Link Diabetes to Thyroid Cancer Risk

Currently, there are some hypotheses linking diabetes to thyroid cancer risk, including (i) increased body mass index (BMI); (ii) elevated insulin levels; (iii) long-term elevation of thyroid-stimulating hormone (TSH); (iv) long-term exposure to high levels of glucose and triglycerides; (v) vitamin D deficiency; (vi) use of antidiabetic medications

including insulin and sulfonylureas [22] (Figure 1). There are some epidemiological studies demonstrating the relationships between thyroid cancer and BMI, TSH, blood glucose, and triglycerides.

3.1. Molecular Pathogenesis of Thyroid Cancer. In normal adults, the weight and composition of the thyroid glands remain generally constant [25]. The cells turnover about 6–8 renewals in adult life [25]. The cell growth is closely regulated by paracrine function of follicular cells, which secrete factors such as insulin-like growth factor I (IGF-1) and fibroblast growth factor to control other cells [26].

In thyroid glands, three distinct mitogenic pathways have been proposed: (i) the hormone receptor adenylate cyclase-cAMP protein kinase A system (AC/cAMP/PKA); (ii) the hormone receptor tyrosine protein kinase (RTK) pathways; (iii) hormone receptor phospholipase C cascade (PLC) pathway [27]. TSH is the major stimulator of the AC/cAMP/PKA pathway by binding to the TSH receptor (TSHR). This pathway regulates the function, differentiation, and proliferation of the thyroid glands [28]. Epidermal growth factor (EGF) stimulates RTK pathway, which leads to an increase in transcriptional activity [29]. The PLC pathway is activated by TSH, neurotransmitters, growth factors, and phorbol ester. This pathway subsequently increases the intracellular calcium and protein kinase C activity [30]. There are two models of thyroid carcinogenesis: fetal cell carcinogenesis theory and multistep carcinogenesis theory [27]. Factors affecting the mitogenic pathways may be involved in the pathogenesis of thyroid cancer.

Molecular pathogenesis involves genetic events [31]. Activating point mutations of the *RAS* genes is frequently found in follicular thyroid carcinomas [31]. Rearrangements of genes (*RET, TKR*) of transmembrane receptors with tyrosine kinase activity and activating point mutations of the *BRAF* gene are found in papillary thyroid carcinomas [31]. Poorly differentiated and anaplastic thyroid carcinomas are found to have inactivating point mutations of the *P53* gene [31]. Mutations of *RET* oncogene may also be responsible for the tumorigenesis of medullary thyroid cancer [31].

3.2. Elevated Insulin Levels and Thyroid Cancer Risk. Chronic elevated circulating insulin level is observed in diabetics and may be due to endogenous (insulin-resistance-related) or exogenous sources (medications). Insulin shares structural homology and affinity of the receptors with IGF-1, and is important for cell proliferation and apoptosis [32]. Elevated insulin and IGF-1 levels are related to various cancers, such as breast and colon cancers [33, 34]. As mentioned above, IGF-1 may control follicular cell growth [26]. In follicular cell cultures, incubation of follicular cells with TSH and insulin causes significant increase in cell number than incubation with TSH alone [35], suggesting that insulin may mimic IGF-1 in follicular cells. Follicular cells do synthesize IGF-1 and have IGF-1 receptors, which is associated with the pathogenesis of thyroid nodules by potentiating TSH action [36]. Therefore, insulin may also play a role in thyroid carcinogenesis. Some studies demonstrated the association between insulin resistance and thyroid nodules and thyroid cancer [37, 38]. However, to our knowledge, there has been no human study directly confirming the association between insulin exposure and thyroid cancer.

3.3. TSH and Thyroid Cancer Risk. As mentioned above, TSH is involved in mitogenic pathways of the thyroid glands [27]. TSH is an independent risk factor of thyroid cancer development [39–41]. Thyroid cancer risk increases with higher TSH level [40]. Higher TSH level is also associated with advanced stage of differentiated thyroid cancer [40]. Diabetic patients are more prone to have chronically mild TSH elevation. Previous study showed that 3% of insulin-dependent diabetics had hypothyroidism, and 13–20% had elevated TSH levels and antithyroid antibodies [42]. A recent study showed that the rate of primary hypothyroidism in type 2 diabetics is greater than in the nondiabetic population (odds ratio = 3.45; 95% CI: 2.51–4.79) [43]. The increased thyroid cancer risk may be related to the elevated TSH level in diabetic patients.

3.4. Increased BMI and Thyroid Cancer Risk. Obesity is associated with several types of cancer, such as adenocarcinoma of the esophagus, colon, kidney, endometrium, and malignant melanoma [44]. Obese people are at a 10-fold increased risk of diabetes [45]; and they may have increased risk of thyroid cancer [22, 46, 47]. Adjustment for BMI slightly reduced thyroid cancer risk associated with diabetes, but BMI only could not explain the association between diabetes and thyroid cancer [22]. Meta-analysis showed that an increase in BMI of $5\,kg/m^2$ was associated with an increased risk of thyroid cancer in both men (RR = 1.33; $P = 0.02$) and women (RR = 1.14; $P = 0.001$) [47].

Potential mechanisms linking obesity and thyroid cancer risk include elevated TSH levels, insulin resistance, and adipokines effect [9, 46]. Some studies showed that BMI and TSH levels were positively correlated, but others did not [46]. As mentioned above, TSH and insulin influence the growth and differentiation of follicular cells [27]. Adipokines such as adiponectin, leptin, and hepatocyte growth factor may regulate cancer cell proliferation and may be related to cancer progression [9]. Increased expression of leptin and its receptor in thyroid cancer were reported [48]. Its association with tumor aggressiveness and biological behavior was also demonstrated [48]. However, an inverse association was identified between BMI and tumor invasion and nodal metastasis in a clinicopathological cohort study [9]. Further study is necessary to determine the relationship between BMI and thyroid cancer outcome.

3.5. Antidiabetic Medications and Thyroid Cancer Risk. According to previous studies, cancer risk in metformin-treated patients is similar to that in patients not receiving medication for diabetes [49]. Metformin diminishes growth stimulation by insulin and inhibits growth of thyroid cancer *in vitro* [50]. There are several mechanisms proposed for the antitumor effect of metformin such as increasing the

AMP-activated protein kinase signaling pathway and a direct influence upon immune competence [51].

Sulfonylureas are associated with increased mortality (HR = 1.3; 95% CI: 1.1–1.6) [52]. Cancer mortality is about doubled among insulin users relative to metformin users (HR = 1.9; 95% CI: 1.5–2.4) [52]. Cancer risk increases by an estimated 20% for each year of insulin therapy [53]. Increased circulating insulin level may be another explanation for the increased cancer risk associated with sulfonylureas and insulin therapy [51]. As mentioned above, high insulin levels and the associated changes of the IGF-1 axis may be associated with cancer development. Glargine, a long-acting insulin analog, may have even higher cancer risks compared with human insulin [51]. This is possibly due to the prolonged binding of IGF-1 receptor, leading to increased mitotic activity [51]. To our knowledge, there has been no human study confirming the association between insulin and sulfonylurea treatment and thyroid cancer [22].

The association of incretin-based therapy and medullary thyroid cancer had been widely discussed. Glucagon-like pepide-1 receptor activation promotes C-cell proliferation and medullary thyroid cancer in rodents [54, 55]. Currently, there is no sufficient data to confirm the association between incretin-based therapies and thyroid cancer in humans [56].

Other potential drugs are peroxisome proliferator-activated receptors (PPARs) γ agonists, which has been demonstrated to promote the growth and invasion of thyroid cancer cells *in vitro* with an increase in G1 phase and a decrease in the S and G2/M phases [57]. But the mechanism is unclear.

3.6. Chronic Glucose and Triglycerides Exposure and Thyroid Cancer Risk. Studies showed that men with elevated level of triglycerides and women with increased blood glucose level were more prone to have thyroid cancer [14, 58, 59]. The possible mechanism is the increased oxidative stress. Free fatty acids and glucose stimulate nuclear factor-κ B, which increases the production of nitric oxide, a substrate for reactive oxygen species (ROS) [60]. Low level of ROS regulates cellular signaling and is important in normal cell proliferation. Increased ROS is observed in cancer cells [14]. However, a recent large-scale cohort analysis shows that glucose was inversely associated with thyroid cancer risk in women below 50 years old, was not related to thyroid cancer risk in women above 50 years old, and was associated with an increased thyroid cancer risk in men [61]. The proposed mechanism is the complex relationship among reproductive hormones, glucose, and thyroid cancer. Since the study lacks detailed information on reproductive history and sex hormone use, it cannot come to any conclusion. In summary, current human studies showed controversial relationship between glucose level and thyroid cancer risk.

3.7. Vitamin D Deficiency and Thyroid Cancer Risk. Vitamin D deficiency is observed in up to 70% of diabetics, although the reason is unclear [22, 62]. Vitamin D promotes differentiation and apoptosis of cancer cells [63]. Low vitamin D level decreases deiodinase 2, resulting in decreased intracellular triiodothyronine (T_3) [22]. Decreased T_3 concentration in skeletal muscle and adipose tissue should lead to decreased glucose transporter 4 transcription and thus lead to insulin resistance [22]. Decreased T_3 concentration in pituitary gland stimulates TSH release [22]. As mentioned above, insulin resistance and TSH may be related to thyroid cancer. To our knowledge, there has been no human study directly confirming the association between vitamin D deficiency and thyroid cancer.

4. Conclusion

Epidemiologic studies showed significant or nonsignificant increases in thyroid cancer risk in diabetic women, and nonsignificant increases or no change in thyroid cancer risk in diabetic men. The results are controversial, and evidence is not strong enough to link diabetes and thyroid cancer. Mechanisms proposed to link diabetes and thyroid cancer include elevated TSH, insulin, glucose, triglycerides, insulin resistance, obesity, vitamin D deficiency, and antidiabetic medications. However, these mechanisms are mostly postulated from epidemiological studies, and studies providing direct biological modes of action are still scarce. Further research is necessary to confirm the relationship between diabetes and thyroid cancer and to explore the underlying mechanisms.

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Clinical Study

Serum CA19-9 Level Associated with Metabolic Control and Pancreatic Beta Cell Function in Diabetic Patients

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CA19-9 is a tumor-associated antigen. It is also a marker of pancreatic tissue damage that might be caused by diabetes. Long-term poor glycemic control may lead to pancreatic beta cell dysfunction which is reflected by elevated serum CA19-9 level. Intracellular cholesterol accumulation leads to islet dysfunction and impaired insulin secretion which provide a new lipotoxic model. This study firstly found total cholesterol was one of the independent contributors to CA19-9. Elevated serum CA19-9 level in diabetic patients may indicate further investigations of glycemic control, pancreatic beta cell function, and total cholesterol level.

1. Introduction

CA19-9 is a tumor-associated antigen that was originally defined by a monoclonal antibody produced by a hybridoma prepared from murine spleen cells immunized with a human colorectal cancer cell line. Although increased serum CA19-9 level is known to be associated with pancreatic cancer. In particular, it has been also shown to increase in many malignant diseases such as upper gastrointestinal tract, ovarian, and hepatocellular and colorectal cancer. In addition, various studies have reported increased serum CA19-9 levels in benign diseases such as inflammatory conditions of the hepatobiliary system, thyroid disease [1], acute and chronic pancreatitis [2], diabetes mellitus [3] (DM), and interstitial pulmonary disease [4].

CA19-9 is used in the diagnosis of pancreatic cancer, but it is also a marker of pancreatic tissue damage that might be caused by diabetes. Benhamou et al. [5] investigated the relationship between the CA19-9 and metabolic control of diabetes in 51 adult patients. They concluded that CA19-9 in diabetic patients is raised in acute metabolic situations, which correlated very well with blood glucose concentration. It was suggested that glucose toxicity may play a role in high serum CA19-9 levels in these patients. Gul et al. [6] showed that serum CA19-9 level was related to microvascular complications in type 2 DM patients.

The aim of this study was to evaluate serum CA19-9 levels in patients with DM in comparison with age- and sex-matched control subjects. In addition, we aimed to find out whether serum CA19-9 level was related with metabolic control and pancreas pancreatic beta cell function in these subjects.

2. Research Design and Methods

2.1. Study Population. 71 type 1 DM, 866 type 2 DM patients, and 122 healthy volunteers who examined and treated in our outpatient clinic and inpatient department were enrolled in this cross-sectional study. The local ethical committee approval was obtained. Patients with malignant disease, with history of chemotherapy or radiotherapy, and with acute or chronic pancreatitis were excluded. Patients with diabetes who have any coexistent disease related to high CA19-9 levels were also excluded. CA19-9 levels were measured in all subjects. Cases with high CA19-9 levels were evaluated with

Table 1: Demographic and clinical characteristics of study subjects.

Variables	Control ($n = 122$)	T1DM ($n = 71$)	T2DM ($n = 866$)	P value
Gender (M/F)	64/58	34/37	507/359	—
Age (y)	47.78 ± 11.23	51.68 ± 17.84$^{*\#\#}$	60.45 ± 11.97**	<0.001
Duration (y)	—	5.00 (1.00–8.00)$^{\#\#}$	8.00 (3.88–13.00)	0.008
ALT (U/L)	18.2 (13.00–26.75)	17.00 (11.00–27.50)	18.0 (13.00–29.00)	0.722
AST (U/L)	21.0 (17.00–25.00)	18.00 (15.00–25.00)	19.0 (15.00–24.00)	0.332
TC (mmol/L)	4.84 ± 0.85	4.60 ± 1.12	4.75 ± 1.20	0.401
TG (mmol/L)	1.20 (0.87–1.79)	0.90 (0.71–1.33)$^{*\#\#}$	1.52 (1.06–2.20)**	<0.001
HDL (mmol/L)	1.35 ± 0.32	1.40 ± 0.42$^{\#\#}$	1.11 ± 0.35**	<0.001
LDL (mmol/L)	3.18 ± 0.87	2.97 ± 1.12	3.17 ± 0.98	0.256
BUN (mmol/L)	4.83 ± 1.21	6.04 ± 2.83**	5.82 ± 2.47**	<0.001
Cr (μmol/L)	67.00 (58.00–78.00)	65.50 (51.25–79.75)	66.00 (54.00–80.00)	0.855
FPG (mmol/L)	5.08 ± 0.34	9.60 ± 4.21$^{**\#\#}$	8.48 ± 3.20**	<0.001
2hPG (mmol/L)	6.06 ± 1.09	14.09 ± 6.17**	14.24 ± 4.76**	<0.001
HbA1c (%)	5.43 ± 0.33	9.81 ± 2.82**	9.34 ± 2.33**	<0.001
GA (%)	—	29.30 ± 8.82$^{\#\#}$	25.94 ± 8.41	0.001
CP0 (ng/mL)	6.84 (5.08–9.85)	0.25 (0.05–0.80)$^{**\#\#}$	1.62 (1.04–2.31)**	<0.001
CP120 (ng/mL)	36.80 (22.44–57.97)	0.40 (0.48–1.42)**	3.37 (2.05–5.29)**	<0.001
ΔCP (ng/mL)	28.84 (16.43–52.42)	0.08 (0.00–0.62)$^{**\#\#}$	1.59 (0.77–2.77)**	<0.001
CA19-9 (KU/L)	4.69 (2.66–9.65)	18.59 (11.68–39.28)$^{**\#\#}$	12.07 (6.72–21.57)**	<0.001

Data represent means ± S.D. or median (interquartile range), $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus T2DM group.
ALT, aspartate aminotransferase; AST, alanine aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; FPG, fasting plasma glucose; 2hPG, 2h plasma glucose; HbA1c, glycated hemoglobin A1C; GA, glycated serum albumin; TG, total triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CP0, C Peptide of 0 min; CP120, C Peptide of 120 min; ΔCP, D value of C Peptide of 120 min minus C Peptide of 0 min.

abdominal ultrasonography and CT imaging. Upper gastrointestinal endoscopy and colonoscopy were performed when needed. Duration of diabetes was calculated by years. Heights and weights of the participants were measured, and their body mass indexes (BMI) [weight (kg)/square of height (m^2)] were calculated.

2.2. Laboratory Tests. Plasma glucose was assayed by glucose oxidase method. Serum C peptide concentration was measured by radioimmunoassay (RIA) (Linco Research, United States). HbA1c was determined by high-performance liquid chromatography (Bio-Rad Inc., Hercules, USA). GA was measured by enzymatic method (LUCICA GA-L, Asahi KASEI, Tokyo). Alanine aminotransferase (ALT) was measured by UV method. Alanine aminotransferase (AST) was measured by Szasz-Persijn method. Serum triglyceride (TG), TC, high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were measured by enzymatic procedures using an autoanalyzer (Hitachi 7600-020, automatic analyzer, Japan). Serum CA19-9 level was measured using chemiluminescence method and access GI monitor kit (Siemens Immulite 2000, Siemens Healthcare Diagnostics; and Immulite 2000, Beckman Coulter, Brea, CA). Normal ranges for serum CA19-9 level were 0 to 35 U/mL, and the levels above higher range were accepted as abnormal.

2.3. Statistical Methods. All analyses were performed with Statistical Package for Social Sciences 11.0 software (SPSS, Chicago, USA). Data were expressed as mean ± SD except skewed variable which was presented as medium (interquartile range 25%–75%), and the data that were not normally distributed were logarithmically transformed before analysis. Clinical characteristics were compared among the three groups using one-way ANOVA test, and several variables without data of control group were compared with independent samples t test. The Pearson and Spearman correlation coefficients were calculated to assess the strength of the correlation of CA19-9 and parameters of glucose and lipid metabolism. The ΔCP represents the difference between the value of CP 120 min and CP 0 min, which regarded as an important indicator of pancreatic beta cell function because many diabetic patients were treated with exogenous insulin. Multiple stepwise regression analysis was performed to determine the associations between serum CA19-9 and metabolic parameters. The variables selected to enter into stepwise regression were those that correlated significantly with serum CA19-9 (after correlation analysis). All reported P values were two-tailed and $P < 0.05$ were considered statistically significant.

3. Results

3.1. Characteristics of Subjects. The general characteristics and clinical parameters of the cross-sectional study are summarized in Table 1. Age, duration of diabetes, TG, HDL-c, blood urea nitrogen (BUN), FPG, 2hPG, HbA1c, GA, CP0, CP120, ΔCP, and CA19-9 level differed significantly among the three groups ($P < 0.01$). People with type 1 and type 2

Table 2: Basic clinical and biochemical characteristics by quartiles of CA19-9.

Variables	CA19-9 quartile				P value
	1 (Lowest)	2	3	4 (Highest)	
Age (y)	56.64 ± 12.198	60.1 ± 12.652*	58.75 ± 13.534	58.21 ± 13.806	0.023
Duration (y)	8.0 (3.0–12.0)	9.0 (4.0–12.5)	8.0 (3.0–13.0)	8.0 (3.0–13.0)	0.327
ALT (U/L)	17.00 (13.00–26.00)	19.00 (13.75–30.00)	17.00 (13.00–75.00)	19.00 (12.00–29.00)	0.398
AST (U/L)	19.00 (15.00–24.00)	19.00 (16.00–24.75)	18.50 (16.00–24.00)	19.00 (16.00–25.00)	0.508
TC (mmol/L)	4.66 ± 0.935	4.62 ± 1.054	4.75 ± 1.201	4.99 ± 1.367**##▲	0.001
TG (mmol/L)	1.45 (0.99–2.08)	1.43 (1.04–2.04)	1.47 (0.93–2.07)	1.44 (0.97–2.21)	0.739
HDL (mmol/L)	1.18 ± 0.428	1.12 ± 0.326*	1.15 ± 0.328	1.18 ± 0.368	0.167
LDL (mmol/L)	3.11 ± 0.849	3.10 ± 0.914	3.16 ± 1.032	3.27 ± 1.086	0.168
BUN (mmol/L)	5.37 ± 1.615	5.84 ± 2.517	5.75 ± 2.340	5.91 ± 2.931	0.056
Cr (μmol/L)	67.00 (57.00–78.25)	68.00 (57.00–81.00)	64.00 (54.00–77.00)	63.00 (51.75–80.50)	0.363
FPG (mmol/L)	6.84 ± 2.367	7.93 ± 2.752**	8.73 ± 3.263**#	9.15 ± 4.116**##	<0.001
2hPG (mmol/L)	10.77 ± 4.70	13.72 ± 4.812**	13.95 ± 5.285**	14.72 ± 5.471**	<0.001
HbA1c (%)	7.36 ± 1.870	8.41 ± 1.945**	9.43 ± 2.444**##	10.49 ± 2.768**##▲▲	<0.001
GA (%)	21.89 ± 5.874	24.30 ± 6.819*	27.18 ± 8.455**##	30.29 ± 9.525**##▲▲	<0.001
CP0 (ng/mL)	2.195 (1.383–4.228)	1.80 (1.18–2.695)*	1.705 (1.04–2.553)**	1.29 (0.64–2.03)**#	<0.001
CP120 (ng/mL)	5.32 (2.85–15.05)	4.21 (2.53–5.82)**	3.27 (1.76–5.44)**#	2.38 (1.31–4.15)**#	<0.001
ΔCP (ng/mL)	2.76 (1.34–9.388)	2.09 (0.94–3.41)**	1.55 (0.53–2.77)**#	1.00 (0.41–2.06)**#▲	<0.001

Data represent means ± S.D. or median (interquartile range), *$P < 0.05$, **$P < 0.01$ versus group 1; #$P < 0.05$, ##$P < 0.01$ versus proup 2; ▲$P < 0.05$, ▲▲$P < 0.01$ versus proup 3.

diabetes had significantly higher FPG, 2hFPG, HbA1c, ΔCP, and CA19-9 level than control group ($P < 0.01$). In addition, there was significant difference between two of the three groups in FPG, CP0, and ΔCP ($P < 0.01$).

3.2. CA19-9 Value Quartile. As is shown in Table 2, the subjects were divided into 4 quartiles on the basis of CA19-9 values. Compared with lower quartile group, the upper quartile group had significant higher FPG, 2hFPG, HbA1c, GA and lower CP0, CP120, and ΔCP ($P < 0.01$). Among all of parameters, HbA1c, GA, and ΔCP had statistic significance in every two groups.

3.3. Correlation Analysis in Groups. In whole participants the correlation analysis (Table 3) showed that serum CA19-9 was positively correlated with TC ($r = 0.129$, $P < 0.001$) FPG ($r = 0.309$, $P < 0.001$), 2hPG ($r = 0.284$, $P < 0.001$), HbA1c ($r = 0.486$, $P < 0.001$), and GA ($r = 0.389$, $P < 0.001$) and was negatively correlated with CP0 ($r = -0.229$, $P < 0.001$), CP120 ($r = -0.365$, $P < 0.001$), and ΔCP ($r = -0.359$, $P < 0.01$).

3.4. Multiple Stepwise Regression Analysis in Groups. To further determine which variables were independently associated with serum CA19-9, multiple stepwise regression analysis was performed (Table 4). We selected the parameters which significantly correlated with serum CA19-9 level showed in Table 3 as independent, serum CA19-9 levels as dependent. As a result, HbA1c, type of diabetes, TC, and ΔCP were independently associated with serum CA19-9 levels.

Table 3: Correlation analysis of serum CA19-9 with variables as follows.

Variables	Serum CA19-9	
	r	P
Age	0.045	0.142
Duration	0.004	0.904
ALT	0.029	0.35
AST	0.038	0.217
TC	0.129**	<0.001
TG	0.048	0.125
HDL	−0.002	0.961
LDL	0.068*	0.028
BUN	0.057	0.07
Cr	−0.064*	0.041
FPG	0.309**	<0.001
2hFPG	0.284**	<0.001
HbA1c	0.486**	<0.001
GA	0.389**	<0.001
CP0	−0.229**	<0.001
CP120	−0.365**	<0.001
ΔCP	−0.359**	<0.001

*$P < 0.05$, **$P < 0.01$.

4. Discussion

This is the first study which demonstrated that increased serum CA19-9 level significantly correlated with serum total cholesterol and pancreatic beta cell function in diabetic patients.

Table 4: Multiple stepwise regression analysis showing variables independently associated with serum CA19-9.

Independent variables enter the model	β	S.E.M	Standardized β	t	P	95% CI for β (lower limit-upper limit)
HbA1c	0.068	0.005	0.433	13.223	<0.001	0.058 to 0.078
Type of diabetes	−0.139	0.043	−0.099	−3.235	0.001	−0.223 to −0.055
TC	0.028	0.009	0.091	3.02	0.003	0.010 to 0.046
ΔCP	−0.019	0.007	−0.084	−2.547	0.011	−0.033 to −0.004

The parameters which significantly correlated with serum CA19-9 level showed in Table 3 were selected to enter into the model.

CA19-9 is a tumor marker mainly used for the diagnosis of pancreatic cancer. However, it is well known that high serum CA19-9 levels can also be found in various diseases, such as nonmalignant obstructive jaundice, thyroid disease, and ovarian diseases. In limited numbers of studies with small sample sizes, patients with diabetes were shown to have increased CA19-9 levels compared with control groups [7, 8]. In our study, we also demonstrated that diabetic patients have increased serum CA19-9 levels than control. Interestingly, we further found that CA19-9 levels in type 1 diabetes were higher than in type 2 diabetes, although there was no significant difference in HbA1c between the two groups.

HbA1c is a marker of chronic glucose toxicity. Significant correlation was also defined between serum CA19-9 levels and HbA1c. In a previous study [9], it was shown that patients with poor glucose control had the highest serum CA19-9 levels. Long-term poorly glycemic control can lead to chronic oxidative stress, which is a central mechanism for glucose toxicity. Our study also demonstrated the positive correlation between the CA19-9 and HbA1c levels. According to CA19-9 value quartile, the upper quartile group had significant higher HbA1c, GA and lower CP0, CP120, ΔCP than the lower quartile group. The multiple stepwise regression analysis also showed that HbA1c was one of the major independent contributors to CA19-9. These results extend those from previous studies and provide additional evidence that long-term poor glycemic control may lead to pancreatic beta cell dysfunction which is reflected by elevated serum CA19-9 level.

The mechanism of increased serum CA19-9 levels in diabetic patients remains unclear. One of them is that the rise of serum CA19-9 level only reflects cellular dysfunction. The lack of insulin could result in a pancreatic exocrine deficiency and release of CA19-9 by ductal cells [10]. Therefore, the increase of serum CA19-9 level might parallel the intensity of cellular functional disorders. Many early studies on pancreatic function in diabetes demonstrated that pancreatic exocrine insufficiency is present in a considerable percentage of patients with diabetes. Autopsy studies and studies on pancreas histology showed marked changes in the exocrine gland in patients with diabetes mellitus as compared to the nondiabetic controls [11]. Blumenthal et al. reported signs of chronic inflammatory changes of the exocrine pancreas in 11.2% of patients with diabetes mellitus as compared to 5.3% in nondiabetic patients [12]. Therefore, the elevated serum CA19-9 level in diabetic patients might be explained by exocrine damaged. In our study, we firstly found that serum CA19-9 level was negatively correlated with ΔC-peptide which reflected the pancreatic beta cell function. Type 1 diabetes is a chronic progressive autoimmune disease, which leads to the loss of pancreatic beta cell. The level of serum CA19-9 in type 1 diabetes was higher than type 2 diabetes. Multiple stepwise regression analysis showed that type 1 diabetes is an independent contributor to CA19-9. These results supported that increased serum CA19-9 levels may well be a biomarker of damaged pancreatic beta cell function.

Diabetes is often accompanied by abnormal blood lipid and lipoprotein levels, but most studies on the link between dyslipidemia and diabetes have focused on TG and free fatty acids (FFAs). More recently, the accumulating data suggested that cholesterol homeostasis is a major regulator of pancreatic beta cell function [13]. Intracellular cholesterol accumulation leads to islet dysfunction and impaired insulin secretion which provide a new lipotoxic model and a potential link of disturbed cholesterol metabolism to impairment of pancreatic beta cell function [14]. Hao et al. [15] indicated that excess cellular cholesterol plays a direct role in islet pancreatic beta cell dysfunction and may be a key factor underlying the progression of type 2 diabetes. Using different animal models, they showed that elevated serum cholesterol leads to increased cholesterol in pancreatic islets. More importantly, islet cholesterol levels directly and significantly impact the extent of glucose-stimulated insulin secretion, independent of FFAs levels. In our literature, multiple stepwise regression analysis showed that TC was one of the independent contributors to CA19-9. This result further indicates that the increased serum TC level may be associated with the decrease of pancreatic beta cell function. It has great implications that the regulation of cholesterol level may be a potential target for therapeutic intervention aimed at preserving or improving pancreatic beta cell function.

The elevated serum CA19-9 level in diabetic patients may indicate further investigations of glycemic control, pancreatic beta cell function, and TC level. One limitation of the present study should be noted that it was a cross-sectional study. A long-term follow-up study of these subjects should be undertaken to further determine the correlation of serum CA19-9 level with pancreatic beta cell function and TC level.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

H. Yu and R. Li contributed equally to this work.

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Research Article

The Influence of Type 2 Diabetes and Glucose-Lowering Therapies on Cancer Risk in the Taiwanese

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Objective. To investigate the association between type 2 diabetes, glucose-lowering therapies (monotherapy with either metformin, sulphonylurea or insulin) and cancer risk in Taiwan. *Methods*. Using Taiwan's National Health Research Institutes database of 1,000,000 random subjects from 2000–2008, we found 61777 patients with type 2 diabetes (age ≥20 years) and 677378 enrollees with no record of diabetes. *Results*. After adjusting for age and sex, we found patients with diabetes to have significantly higher risk of all cancers (OR: 1.176; 95% CI: 1.149–1.204, $P < 0.001$). Diabetic patients treated with insulin or sulfonylureas had significantly higher risk of all cancers, compared to those treated with metformin (OR: 1.583; 95% CI: 1.389–1.805, $P < 0.001$ and OR: 1.784; 95% CI: 1.406–2.262, $P < 0.001$). Metformin treatment was associated with a decreased risk of colon and liver cancer compared to sulphonylureas or insulin treatment. Sulfonylureas treatment was associated with an increased risk of breast and lung cancer compared to metformin therapy. *Conclusions*. Taiwanese with type 2 diabetes are at a high risk of breast, prostate, colon, lung, liver and pancreatic cancer. Those treated with insulin or sulfonylureas monotherapy are more likely to develop colon and liver cancer than those treated with metformin.

1. Introduction

Cancer has become the leading cause of death in Taiwan since 1982 [1]. Tseng [2] reported cancer to be the second leading cause of mortality in patients with type 2 diabetes in Taiwan. Patients with type 2 diabetes are known to be at increased risk of cancer and cancer mortality [3–9], especially hepatic [3] pancreatic [6], colon [8], bladder [9–11], and breast cancer [5, 12]. The relationship between type 2 diabetes and cancer is complex, possibly involving insulin resistance, hyperinsulinemia, and elevated levels of insulin-like growth factor-1 (IGF-1) in tumor cell growth [13, 14].

Glucose-lowering therapy may also play a role in the relationship between type 2 diabetes and cancer. Metformin treatment might reduce the risk of tumor development [15–20], whereas insulin and sulphonylureas might increase the risk [21, 22]. This study used Taiwan's National Health Insurance claims database to investigate the relationship between type 2 diabetes, glucose-lowering therapy with either metformin, sulphonylureas, or insulin alone, and cancer in the Taiwanese.

2. Methods

2.1. Data Sources. Taiwan's National Health Insurance (NHI) medical claims database, including ambulatory care, hospital inpatient care, dental services, and prescription drugs, was provided by Taiwan's National Health Research Institutes (NHRI). NHI coverage rate was 96.16% of the whole population in 2000 and rose to 99% by the end of 2004. The data set used for this study was a randomly sampled cohort of 1 million individuals enrolled in the NHI system

from 2000 to 2008. It included information on registration entries, ambulatory care claims, inpatient care claims, and prescription. Patient identification numbers were scrambled for protection of confidentiality, and hence no ethics board approval was needed.

NHI diagnosis coding follows the International Classification of Diseases, Ninth Revision (ICD-9), Clinical Modification diagnostic criteria. Records of claims for diabetes care were collected for patients with diabetes-related diagnosis with ICD-9 code 250 (excluding type 1 diabetes with ICD-9 code 250.1). An individual was classified as a diabetic patient if she or he had an initial diabetes-related diagnosis at any time in 2000 and then had at least one service claim from either ambulatory or inpatient care within the subsequent twelve months. Focusing on newly diagnosed cancer cases, we excluded patients diagnosed for any type of cancer (ICD-9: 140–209, 230–239) before the first year of the study period (2000-2001). The end of study period for each enrollee, both diabetic and nondiabetic, was, if any, first episode of primary or secondary diagnosis of any types of cancer from 2002 to 2008.

The previous studies revealed that metformin treatment [15–20] might reduce the risk of tumor development and insulin and sulphonylurea [21, 22] might increase it. Our study wanted to clarify the influence of these antidiabetic agents (metformin, insulin, and sulphonylurea) on cancer risk in Taiwanese. To investigate the association between antidiabetic agents (monotherapy with either metformin, sulphonylurea, or insulin) and incident cancer, we included only type 2 diabetic patients receiving monotherapy with either metformin, sulphonylurea, or insulin. The kind of insulin included intermediate/long acting human insulin (HI), insulin glargine, insulin detemir, fast acting HI and insulin analogues, and premix HI and insulin analogues. The kind of sulphonylurea included glibenclamide, gliclazide, glipizide, and glimepiride. These patients had to have received continuous drug coverage for at least one year during study period and have no prior diagnosis of cancer. We excluded patients who were diagnosed as having cancer before the time they were prescribed antidiabetic drugs during the study period. The male patients were excluded from our analysis of breast cancer, and the female patients from our analysis of prostate cancer.

2.2. Statistical Analysis. The risk of type 2 diabetes on cancers was tested by logistic regression models with age and sex adjustment. To determine the independent effects of antidiabetic drugs on the risk of any types of cancers, we used logistic regression models with age and sex adjustment. Breast cancer and prostate cancer were only adjusted for age but not sex. All statistical operations were performed using SAS version 9.2. A P-value of less than 0.05 was considered significant.

3. Results

In total, 61777 patients with type 2 diabetes (mean age 61.44 ± 13.23 years; 51.1% male) were followed up from 2000 to 2008. Patients with type 2 diabetes were found to be at a significantly higher risk of all cancers (odds ratio (OR): 1.176; 95%, confidence interval (CI): 1.149–1.204, $P < 0.001$) after adjusting for sex and age while compared to nondiabetic subjects (Table 1). Female and elderly subjects were at a significantly higher risk of all cancers than their male and younger counterparts (OR: 1.293, 95% CI: 1.273–1.313, $P < 0.001$ and OR: 1.040, 95% CI: 1.039–1.040, $P < 0.001$, resp.).

Table 1: Adjusted odds ratios and 95% confidence intervals for all cancers associated with type 2 diabetes, sex, and age.

Covariate	Adjusted odds ratio	95% CI	P value
Type 2 diabetes (versus nondiabetic subjects)	1.176	1.149–1.204	<0.001
Sex (female versus male)	1.293	1.273–1.313	<0.001
Age (every 1-yr increment)	1.040	1.039–1.040	<0.001

As can be seen in Table 2, a summary of incident cases of different types of cancer in patients with and without diabetes, patients with type 2 diabetes were at significantly higher risk of breast cancer, prostate cancer, colon cancer, lung cancer, liver cancer, and pancreatic cancer after adjusting for sex and age as compared to nondiabetic subjects.

A total of 10189 patients with type 2 diabetes (mean age 61.18 ± 14.03 years; 52.2% male) were identified as receiving monotherapy of insulin, sulfonylureas, or metformin. Patients receiving insulin or sulfonylurea had a higher risk of all cancers, compared to those receiving metformin (OR: 1.583, 95% CI: 1.389–1.805, $P < 0.001$ and OR: 1.784, 95% CI: 1.406–2.262, $P < 0.001$, resp.), after adjusting for sex and age (Table 3). Female patients with type 2 diabetes were at a significantly lower risk of all cancers than the male patients with type 2 diabetes (OR: 0.777, 95% CI: 0.692–0.873, $P < 0.001$). Elderly patients with type 2 diabetes were at a significantly higher risk of all cancers (OR: 1.037, 95% CI: 1.033–1.042, $P < 0.001$) as compared to younger patients with type 2 diabetes.

Table 4 shows the adjusted odds ratios for specific cancers associated with antidiabetic drugs. Patients receiving insulin or sulphonylureas had a higher risk of colorectal and liver cancers compared to those receiving metformin after adjusting sex and age. Sulphonylureas were additionally associated with an increased risk of breast and lung cancer. We found no relationship between glucose-lowering therapy and prostate, esophageal, gastric, or pancreatic cancer.

4. Discussion

Our study demonstrates that Taiwanese with type 2 diabetes are at a high risk of cancer, especially breast, prostate, colon, lung, liver, and pancreatic cancer compared to nondiabetic subjects (Tables 1 and 2). Among diabetic patients, those receiving insulin or sulphonylurea monotherapy are at a higher risk of cancer compared to those receiving metformin (Table 3). Patients treated with metformin are at a lower risk

Table 2: Adjusted odds ratios and 95% confidence intervals for specific types of cancer associated with type 2 diabetes.

Site-specific cancer	Patients with type 2 diabetes ($n = 61777$)		Subjects without diabetes ($n = 677378$)		Adjusted odds ratio (95% CI)		P value
	Cases	%	Cases	%			
Breast	665	2.20	4042	1.16	1.111	(1.018–1.212)	0.018
Prostate	587	1.86	2223	0.68	1.137	(1.036–1.249)	0.007
Colon	1739	1.98	7219	1.07	1.206	(1.142–1.274)	<0.001
Lung	1226	2.81	4281	0.63	1.296	(1.214–1.384)	<0.001
Liver	1528	2.47	5558	0.82	1.582	(1.491–1.680)	<0.001
Stomach	523	0.85	2700	0.40	0.920	(0.836–1.012)	0.088
Pancreas	286	0.46	731	0.11	2.038	(1.768–2.349)	<0.001

Subjects without diabetes as reference and adjustment for sex and age.

Table 3: Adjusted odds ratios and 95% confidence intervals for all cancers associated with antidiabetic drugs, sex, and age in type 2 diabetic patients treated with metformin, sulfonylurea, or insulin monotherapy.

Covariate	Adjusted odds ratio	95% CI	P value
Antidiabetic drugs			
Sulfonylurea versus metformin	1.784	1.406–2.262	<0.001
Insulin versus metformin	1.583	1.389–1.805	<0.001
Sex (female versus male)	0.777	0.692–0.873	<0.001
Age (every 1-yr increment)	1.037	1.033–1.042	<0.001

of colorectal and liver cancers, compared to those receiving either insulin or sulphonylurea (Table 4), and at a lower risk of breast and lung cancer (Table 4), compared to those receiving sulphonylureas.

Type 2 diabetes has already been linked to an increased risk of cancer [3–12]. One meta-analysis [5] found the relative risk (RR) of breast cancer to be 1.20 for women with diabetes compared to women without diabetes. Three meta-analyses found RR of colon, pancreatic, and hepatocellular cancer in diabetic patients to be 1.30 [8], 1.82 [6], and 1.84 [3], compared to nondiabetic subjects. However, the incidence rates of type 2 diabetes and cancer vary widely across populations. The current study found people with diabetes in Taiwan to be at a high risk of all cancers (odds-ratio (OR): 1.176; 95%, confidence interval (CI): 1.149–1.204, $P < 0.001$), especially breast, colon, liver, lung, prostate, and pancreatic cancers (Table 2). Recently, Lee et al. [23] also reported patients with diabetes to be at a high risk of liver, colon, lung, and prostate cancer. Our study found that patients with type 2 diabetes were not at high risk of gastric cancer (Table 2). Recently, Tseng [24] reported that diabetic Taiwanese have a higher risk of gastric cancer mortality. It should be pointed out that overall incidence of gastric cancer and mortality from the disease are two different entities and probably linked to different factors.

Previous studies [25–28] have shown that cancer incidence is much higher in males than females at nearly all ages. Our study revealed that female Taiwanese have higher incidence of cancers as compared to male subjects in the general population (Table 1). However, we found that female diabetic patients have lower incidence of cancers as compared to male diabetic patients (Table 3). The sex disparities in cancer incidence might be due to illness behavior, health care access and utilization and ethnic difference [29, 30]. Future epidemiologic studies should be encouraged to design, analyze, and report sex-specific associations to aid the understanding of sex differences in cancer incidence in Taiwanese.

The association between diabetes and cancer may be mediated by metabolic syndrome and obesity through hyperinsulinemia and insulin resistance. Insulin is a growth hormone and is known to have atherogenic and mitogenic properties [31–33]. One observational study showing a relationship between level of circulating insulin and cancer has suggested that cancer growth may be influenced by the insulin-IGF-1 signaling axis [34].

This study found male Taiwanese with diabetes to be at a higher risk of prostate cancer than nondiabetic subjects (OR: 1.137, 95% CI: 1.036–1.249, $P = 0.007$, Table 2). This is inconsistent with Tseng's finding of a positive association between diabetes and prostate cancer in Taiwan, an association that became more remarkable in the younger patients [35]. However, previous studies of populations with European ancestries [36, 37] have reported men with diabetes to have a 20% lower risk of developing prostate cancer than men without diabetes. Two large-scale population-based cohort studies in Japan [38, 39] found no relationship between diabetes and prostate cancer, and one recent study [40] has suggested that diabetes is a protective factor for prostate cancer across populations, including Japanese Americans. The possible reasons for these inconsistent results may be ethnic and environment factors, screening frequency of prostate cancer, and the use of prostate specific antigen.

Our study also found that diabetic Taiwanese were at a high risk of lung cancer (OR: 1.296, 95% CI: 1.214–1.384, $P < 0.001$, Table 2). Coughlin et al. [4] reported diabetic men as well as women to be at a higher risk of lung cancer in the US. Jee et al. [41], studying UK population, also found slightly higher but insignificant risk of lung cancer for men and significantly higher risk for women,

Table 4: Adjusted odds ratios and 95% confidence intervals for specific cancers associated with antidiabetic drugs.

Site-specific cancer	Metformin ($n = 3963$)		Sulfonylurea ($n = 6072$)		Insulin ($n = 751$)		Age-sex-adjusted odds ratio (95% CI)	Age-sex-adjusted odds ratio (95% CI)
	Cases/n	%	Cases/n	%	Cases/n	%	Insulin versus metformin	Sulfonylureas versus metformin
Breast*	19/2048	0.93	48/2804	1.71	5/338	1.48	1.630 (0.604–4.396)	1.765 (1.030–3.024)
Prostate*	27/1915	1.41	52/3268	1.59	5/413	1.21	0.893 (0.338–2.359)	1.034 (0.644–1.659)
Colon and rectum	46/3963	1.16	145/6072	2.39	18/751	2.40	2.135 (1.226–3.717)	1.847 (1.320–2.585)
Lung	45/3963	1.14	122/6072	2.01	9/751	1.20	1.058 (0.513–2.183)	1.570 (1.110–2.220)
Liver	58/3963	1.46	143/6072	2.36	19/751	2.53	1.818 (1.075–3.077)	1.504 (1.104–2.049)
Stomach	20/3963	0.50	54/6072	0.89	7/751	0.93	1.855 (0.779–4.419)	1.547 (0.923–2.594)
Pancreas	8/3963	0.20	21/6072	0.35	1/751	0.13	0.693 (0.087–5.545)	1.594 (0.705–3.619)

* Breast cancer and prostate cancer were only adjusted for age but not sex.

after adjusting for age, age squared, smoking, and drinking. Some cohort studies, however, have reported a negative association between diabetes and lung cancer [42–44]. Given these inconsistent findings, further prospective studies are needed to confirm the relationship between type 2 diabetes and specific cancers in different ethnic populations.

Our study found that diabetic patients treated with sulphonylurea or insulin monotherapy were at a significantly higher risk of cancers, compared to those treated with metformin. Likewise, Currie et al. [45] have also found diabetic patients on insulin or insulin secretagogues to be more likely to develop solid cancers than those on metformin. Bowker et al. [46] reported that patients with type 2 diabetes treated with sulphonylureas and insulin are at significantly increased risk of cancer-related mortality than those treated with metformin. Our finding that not only treatment with insulin but also treatment with sulphonylurea increased the risk seems to exclude an adverse property of the insulin formulation itself. Our study revealed that there was no significant association between antidiabetic agents (monotherapy with either metformin, sulphonylurea, or insulin) and prostate cancer in Taiwanese with type 2 diabetes. Tseng [47] also reported that insulin use is not significantly predictive for prostate cancer mortality in diabetic Taiwanese. Recently, Lai et al. [48] also reported that the use of metformin would decrease the risk of lung cancer in Taiwanese with diabetes. Taken together, these studies strongly suggest that glucose-lowering agents may play a role in the relationship between type 2 diabetes and some cancers. How they do this remains unclear. Metformin decreases insulin resistance and may thus possibly reduce the risk of cancer. It might also directly act on AMP-activated protein kinase (AMPK) signaling pathway [17].

Compared to those treated with sulphonylureas or insulin, diabetic patients treated with metformin had a significantly lower risk of developing colorectal and liver cancers (Table 4). Currie et al. [45] also found an association between metformin use and a lower risk of colon cancer. We further revealed that metformin was associated with a lower risk of breast and lung cancer, compared to sulphonylureas (Table 4). These findings suggest that metformin may have anticancer effects, sufficient to justify its use as a first-line treatment for diabetes and its potential use outside the context of diabetes.

This study has several limitations. First, it is subject to many limitations inherent to all observational studies. We lacked information on potentially important clinical covariates, such as smoking status, weight or body mass index, glycemic control, and alcohol consumption. Second, patients might be prescribed different treatment regimens for health-related reasons. Third, there were relatively small numbers of some specific cancers in patients with different glucose-lowering therapy, thereby limiting the power of our analysis.

In conclusion, type 2 diabetes is a risk factor for cancer in the Taiwanese. Metformin use was associated with a lower risk of cancer of the colon and liver, two common cancers in Taiwan. Further prospective studies are needed to confirm this relationship and study the possible mechanisms between cancers and antidiabetic drugs in Taiwan.

Authors' Contribution

M.-C. Hsieh and T.-C. Lee Contributed equally to the paper.

Conflict of Interests

The authors declared that they have no conflict of interrests.

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Review Article

Insulin Resistance and Cancer Risk: An Overview of the Pathogenetic Mechanisms

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Insulin resistance is common in individuals with obesity or type 2 diabetes (T2D), in which circulating insulin levels are frequently increased. Recent epidemiological and clinical evidence points to a link between insulin resistance and cancer. The mechanisms for this association are unknown, but hyperinsulinaemia (a hallmark of insulin resistance) and the increase in bioavailable insulin-like growth factor I (IGF-I) appear to have a role in tumor initiation and progression in insulin-resistant patients. Insulin and IGF-I inhibit the hepatic synthesis of sex-hormone binding globulin (SHBG), whereas both hormones stimulate the ovarian synthesis of sex steroids, whose effects, in breast epithelium and endometrium, can promote cellular proliferation and inhibit apoptosis. Furthermore, an increased risk of cancer among insulin-resistant patients can be due to overproduction of reactive oxygen species (ROS) that can damage DNA contributing to mutagenesis and carcinogenesis. On the other hand, it is possible that the abundance of inflammatory cells in adipose tissue of obese and diabetic patients may promote systemic inflammation which can result in a protumorigenic environment. Here, we summarize recent progress on insulin resistance and cancer, focusing on various implicated mechanisms that have been described recently, and discuss how these mechanisms may contribute to cancer initiation and progression.

1. Introduction/General Overview

Insulin resistance is a pathological condition in which insulin action is impaired in peripheral target tissues including skeletal muscle, liver, and adipose tissue. Initially, in individuals destined to develop T2D, the pancreatic beta cells increase insulin production to overcome insulin resistance and maintain euglycemia. Frank T2D in insulin-resistant individuals develops when beta cells fail to compensate [1, 2]. Also, insulin resistance is a cardinal feature of the metabolic syndrome, a quartet of vascular risk factors which include, in addition to insulin resistance, central obesity, dyslipidemia, and systemic hypertension [3]. With the exception of rare, monogenic forms of insulin resistance, common insulin resistance is a very heterogeneous disorder for which both genetic and environmental factors jointly determine susceptibility [4]. The environmental component reflects the unfavorable global shift toward a western lifestyle of overeating and sedentary habits, with obesity as the outcome [2, 5]. The genetic factor is linked to quantitative and/or qualitative defects in the insulin receptor (INSR) signaling pathway which regulates growth and metabolic responses to insulin, in insulin target cells and tissues [6]. Patients with insulin resistance show an increased morbidity and mortality, largely attributable to cardiovascular disease and T2D [7, 8]. Moreover, a number of epidemiological studies have consistently demonstrated that the risk for several types of cancer (including that of the breast, colorectum, liver, and pancreas) is higher in insulin-resistant patients [9]. As illustrated in Figure 1, various mechanisms have been

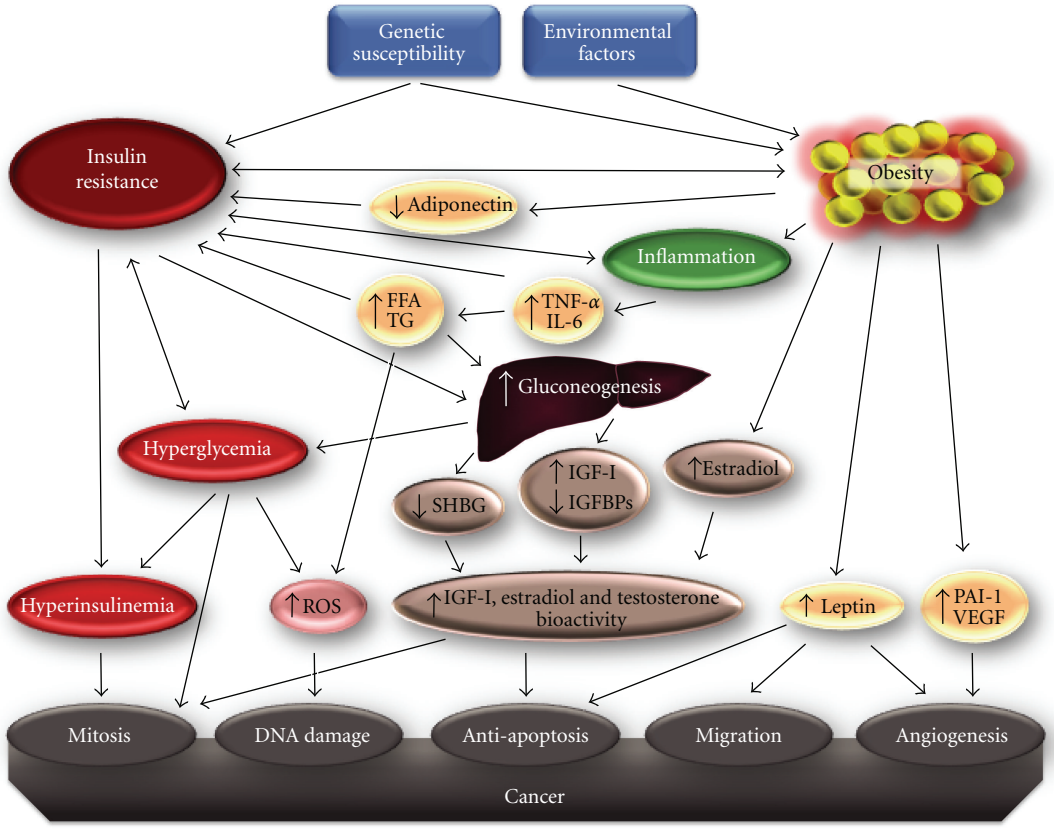

Figure 1: A multidimensional model of cancer development, which suggests insulin resistance and inflammation as driving forces behind cancer. TG: triglycerides; FFA: free fatty acids; TNF-α: tumor necrosis factor α; IL-6: interleukin-6; ROS: reactive oxygen species; SHBG: sex-hormone-binding globulin; IGF-I: insulin-like growth factor I; PAI-1: plasminogen activator inhibitor-1; IGFBPs IGF-I binding proteins; VEGF, vascular endothelial growth factor.

proposed to explain this link, although a complete picture is yet to emerge. The following is a summary of major specific issues currently under debate, related to this area of research.

(1) Chronic hyperinsulinemia, in affected individuals, may promote cancer, as insulin can exert its oncogenic potential via abnormal stimulation of multiple cellular signaling cascades, enhancing growth factor-dependent cell proliferation and/or by directly affecting cell metabolism.

(2) Insulin increases the bioactivity of IGF-I by enhancing hepatic IGF-I synthesis and by reducing hepatic protein production of the insulin-like growth factor binding proteins 1 (IGFBP-1) and 2 (IGFBP-2) [10, 11]. Therefore, although insulin can directly induce tumour growth, many of its mitogenic and antiapoptotic effects are operating through the IGF-I system, as reported in individuals with high levels of circulating IGF-I, in which an increased risk of developing certain types of tumours, in particular breast and prostate cancers, has been documented [12, 13].

(3) Insulin, by reducing SHBG levels, exerts a positive effect on estrogen bioavailability, therefore increasing breast cancer risk.

(4) Obesity, the most common cause of insulin resistance, is increasingly recognized as a low-grade inflammatory state in which overproduction of certain molecules, such as free fatty acids, interleukin-6 (IL-6), adiponectin, leptin, tumour necrosis factor alpha (TNF-α), plasminogen activator inhibitor-1, and monocyte chemoattractant protein (MCP-1), can play a role in malignant transformation and/or cancer progression [14]. In this context, chronic hyperglycemia and increased oxidative stress may also contribute to increased cancer risk.

Therefore, many lines of evidence support the concept that a relationship exists between insulin resistance and cancer, although further studies must be done before this relationship can be fully understood.

2. The INSR, Biological Function, and Its Clinical Significance in Cancer

The first step in insulin action is its interaction with the INSR, an integral membrane glycoprotein with intrinsic enzymatic activity. The INSR belongs to the tyrosine kinase growth factor receptor family and functions as an enzyme that transfers phosphate groups from ATP to tyrosine

residues on intracellular target proteins, thus playing a critical role in both directing the hormone to a specific target tissue and programming the biological response of the tissue to the hormone. The INSR consists of two identical extracellular α subunits (130 kDa) that house insulin binding domains and two transmembrane β subunits (95 kDa) that contain ligand-activated tyrosine kinase activity in their intracellular domains [15–18]. Upon binding of insulin to the α subunits, the receptor becomes activated by tyrosine autophosphorylation, and then the INSR tyrosine kinase phosphorylates various intracellular effector molecules (e.g., IRS proteins and Shc) which in turn alter their activity, thereby generating a biological response [16–19]. The INSR exists as two splice variant isoforms: the INSR-B isoform that is responsible for signaling metabolic responses involved mainly in the regulation of glucose uptake and metabolism and the INSR-A isoform that is expressed in certain tumours (such as mammary cancers), signals predominantly mitogenic responses, and is capable of binding IGF-II with high affinity [20, 21]. As a consequence of these cellular activities, abnormalities of INSR expression and/or function can facilitate the development of several metabolic and neoplastic disorders. Abnormalities in the INSR signaling pathway are implicated in certain common dysmetabolic disorders, including obesity, T2D, the metabolic syndrome, and the polycystic ovary syndrome [22–25]. Also, rare clinical syndromes due to mutations in the *INSR* gene have been identified in patients with monogenic forms of severe insulin resistance [26, 27]. A relation between INSR and cancer has been established following the observation that overexpression of functional INSRs can occur in human breast cancer and other epithelial tumours, including ovarian and colon cancer, in which the INSR may exert its oncogenic potential via abnormal stimulation of multiple cellular signaling cascades, enhancing growth factor-dependent proliferation, and/or by directly affecting cell metabolism [28–33]. On the other hand, epidemiological and clinical evidence points to a link between insulin-resistant syndromes, such as obesity and T2D, and cancer of the colon, liver, pancreas, breast and endometrium. The mechanistic link between insulin resistance and cancer is unknown, but constitutive activation of the tyrosine kinase activity of INSR and related downstream signaling pathways by chronic sustained hyperinsulinemia, in these clinical syndromes, appears to have a role in the neoplastic transformation process [34–36]. Mechanisms due to hyperinsulinemia that promote malignancy and neoplastic progression include the increase in IGF-I and sex hormones bioavailability, the increase in proinflammatory cytokines, and oxidative stress. Although the molecular mechanisms that cause neoplastic transformation, and sustain tumour progression in the presence of INSR hyperexpression and/or hyperstimulation are not fully understood, an explanation for increased INSR expression in epithelial tumours has been recently provided by our group in both breast cancer cell lines and human breast cancer tissues, in which overexpression of the nuclear transcription factor activator protein 2-α (AP2-α) accounted for INSR overexpression [37] (Figure 2(a)). In these cases, we demonstrated that transactivation of the *INSR* gene by AP2-α represented a fundamental prerequisite to activate *INSR* gene transcription in neoplastic breast tissue. Similarly, a functional link between INSR and cyclin D1 has been recently described in pancreatic cancer [38]. Thiazolidinediones (TZDs), a class of commonly used antidiabetic drugs that act as peroxisome proliferator-activated receptor (PPARγ) agonists, have shown antiproliferative effects in many studies *in vitro* and *in vivo* and have been therefore proposed as an auxiliary anticancer therapy in some clinical trials [39]. Recently, we showed that *INSR* gene transcription and protein expression were reduced in cells with forced expression of PPARγ or TZD-induced PPARγ activation (Figure 2(b)). These findings were confirmed in MCF-7 human breast cancer cells overexpressing PPARγ, and 3T3-L1 adipocytes producing relatively high amounts of endogenous PPARγ [40, 41]. Molecular biology studies using GST pull-down, combined with electrophoresis mobility shift assay and chromatin immunoprecipitation, have demonstrated that, in selected cell lines, PPARγ physically interacts with Sp1, AP2-α, and C/EBPβ, preventing binding of AP2-α to Sp1, as well as binding of Sp1 and C/EBPβ to their DNA consensus sites within the *INSR* gene locus [42]. Therefore, it has been postulated that PPARγ may perturb *INSR* gene expression by interfering with the transcriptional initiation complex during activation of the *INSR* gene. This observation might contribute to the identification of new therapeutic targets for treatment of tumours in which abnormal expression and/or function of INSR occur.

The INSR can be regulated by a wide variety of factors and under different environmental conditions [43]. For example, glucocorticoids enhance transcription of the *INSR* gene, whereas insulin downregulates its own receptor. As a step toward understanding the molecular basis of regulation of *INSR* gene expression, the promoter region of the human *INSR* gene has been first identified and then analyzed by several groups [44–46]. This region extends over 1800 bases upstream from the *INSR* gene ATG codon and is extremely GCrich, containing a series of GGGCGG repeats that are putative binding sites for the mammalian transcription factor Sp1. It has neither a TATA box nor a CAAT box, reflecting the common features for the promoters of constitutively expressed genes (so-called housekeeping genes). The INSR is expressed at higher levels in differentiated target tissues such as muscle and fat. At these levels, tissue-specific and ubiquitous nuclear transcription factors cooperate to induce *INSR* gene transcription. We have previously identified two distinct, functionally active DNA sequences, C2 and E3, within the *INSR* gene promoter, which had a significant ability to drive *INSR* gene transcription [46]. The molecular mechanisms regulating *INSR* gene expression have been widely studied by our group and evidence has been provided showing that the architectural transcription factor high-mobility group A1 (HMGA1) is required for proper transcription of the *INSR* gene. HMGA1 is a small basic protein that binds to AT-rich regions of certain gene promoters and functions mainly as a specific cofactor for gene activation [47–49]. HMGA1 by itself has no intrinsic transcriptional activity; rather, it has been shown to transactivate promoters through mechanisms that facilitate the assembly and stability

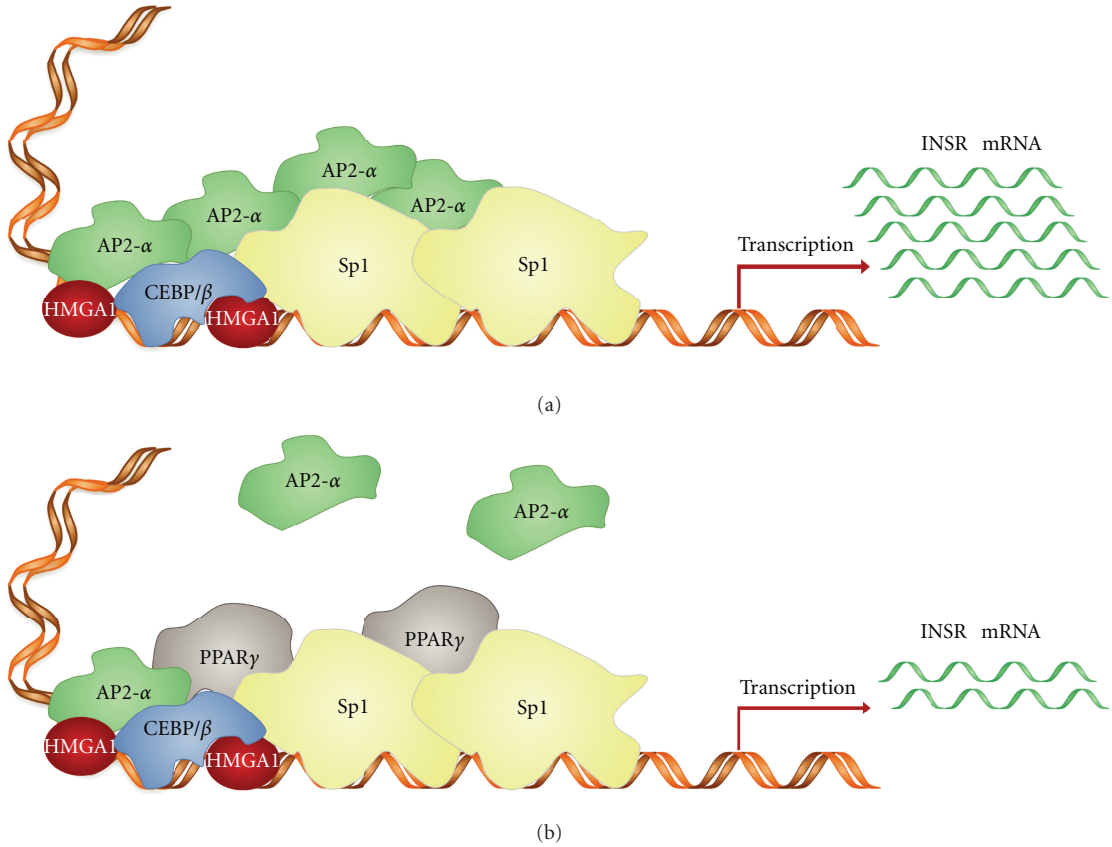

Figure 2: *INSR* gene expression in breast cancer. (a) AP2-α overexpression increases INSR expression in breast tumour [37]. Transactivation of the *INSR* gene by AP2-α occurs indirectly through physical and functional cooperation with HMGA1 and Sp1. (b) By binding to AP2-α and Sp1, PPARγ and agonists may attenuate the stimulatory effect of AP2-α on *INSR* gene transcription in breast cancer.

of stereospecific DNA-protein complexes, "enhanceosomes," that drive gene transcription. HMGA1 performs this task by modifying DNA conformation and by recruiting transcription factors to the transcription start site, facilitating DNA-protein and protein-protein interactions [47–49]. By potentiating the recruitment and binding of Sp1 and C/EBPβ to the *INSR* promoter sequence, HMGA1 greatly enhances the transcriptional activities of these factors in this gene context [46, 50, 51]. Qualitative and/or quantitative defects in these binding proteins and/or abnormalities in their consensus sequences within the *INSR* gene may affect *INSR* gene transcription, leading to abnormalities in *INSR* gene and protein expression [26]. Overexpression of INSR in cells which normally express low levels of INSR, like epithelial cells, may increase the biological responses to insulin and trigger a ligand-mediated neoplastic transformation. Various studies have shown that INSRs are increased in most human breast cancers, and both ligand-dependent malignant transformation and increased cell growth occur in cultured breast cells overexpressing the INSR [37, 52, 53]. Also, overexpression of functional INSRs has been involved in thyroid carcinogenesis [54]. In all these cases, the INSR can exert its oncogenic potential in malignant cells via abnormal stimulation of multiple cellular signaling cascades, enhancing growth factor-dependent proliferation and/or by directly affecting cell metabolism.

3. Proposed Mechanisms for Hormone-Mediated Tumorigenesis

Chronic hyperinsulinemia in insulin-resistant patients increases bioavailability of IGF-I by reducing hepatic gene expression and protein production of IGFBP-1 and IGFBP-2. Also, a decrease in circulating levels of SHBG, followed by an increase in the bioavailability of estradiol and testosterone, may occur in these patients, in whom the combined effect of increased synthesis and bioavailability of estradiol and testosterone can have an adverse impact on target cells and tissues expressing estrogen and androgen receptors. The effect of sex steroid binding to their specific receptors can vary, depending on tissue type, but in some tissues (e.g., breast epithelium and endometrium), this hormone-receptor interaction results in abnormal cellular proliferation and inhibition of apoptosis. Of major importance in hormone-mediated cancers is the IGF system. This system is composed of at least three ligands (insulin, IGF-I, and IGF-II), two receptors (IGF-IR and INSR) and six structurally similar IGFBPs that have important influence over the biological effectiveness of the IGFs, since they are able to increase the half-lives of circulating IGFs, hence controlling their availability for receptor binding [55]. IGFBP-3 is the predominant binding protein expressed in serum, and the vast majority of circulating IGF-I and IGF-II are bound in

a ternary complex with IGFBP-3 and a third component, the acid-labile subunit. In addition, IGFBP-3 directly regulates the interaction of IGF-I with its receptor and, through IGF-independent mechanisms, is able to inhibit cell growth and induce apoptosis. The primary location for IGFBP-3 production is in the liver, where its expression is upregulated by the growth hormone (GH) and suppressed by insulin. Because of the IGF-I's mitotic properties, lower levels of IGFBP-3, by increasing the IGF-I/IGFBP-3 ratio, may increase the risk of developing cancer, with the opposite occurring when tissue availability of IGF-I is reduced. Like IGFBP-3, the biosynthesis of IGF-I occurs primarily in the liver, where its production is GH dependent [56–58], and is increased by insulin [56, 57]. Low insulin levels, as encountered in individuals with type 1 diabetes, or following a prolonged fasting state, by determining the reduction of GH receptor expression, can contribute to lowering the hepatic IGF-I protein synthesis, thus reducing circulating levels of IGF-I. The reduced bioavailability of IGF-I under these conditions is accompanied by an increase in circulating levels of IGFBP-1 and IGFBP-2, the expression of both of which is normally suppressed by insulin. Consistently, higher expression of GH receptors with increased IGF-I protein production can be detected in patients with sustained hyperinsulinemia and T2D [59]. On the other hand, less IGFBP expression following malignant transformation has been reported in some tumour cell types in which the amount of free IGF-I may, therefore, increase even if there is no change in the rate of IGF-I production [60].

The IGF-IR is homologous to the INSR (sharing 84% amino acid identity in the intracellular tyrosine kinase domains). Because of their high sequence similarity [61, 62], an INSR hemireceptor may assemble with an IGF-IR hemireceptor, forming INSR/IGF-IR hybrid receptors. It has been demonstrated that signaling through these receptors regulates cell survival and proliferation [63, 64]. Both insulin and IGF-I bind to the extracellular α subunits of their cognate receptors and induce conformational changes that cause the activation of the tyrosine kinase domain and self-phosphorylation of tyrosine residues of the intracellular β subunit [65]. The INSR, the IGF-IR, as well as the hybrid receptors, are expressed at higher levels in malignant cells [66]. Functional activation of these receptors results in the upregulation of the INSR substrate 1 (IRS1), that triggers signaling pathways downstream of the mitogenic-activated protein (MAP) kinase pathway and the phosphoinositide-3 kinase/Akt (PI3K/Akt), two of the most important signaling cascades frequently dysregulated in cancer (Figure 3). PI3K is recruited to the membrane after being activated by growth factors and cytokines. At this level, the enzyme is activated and transfers a phosphate group to its substrate, phosphatidylinositol [4, 5]-bisphosphate [PtdIns(4,5)*P*2], forming PtdIns-(3,4,5)-*P*3 [67]. The PtdIns(3,4,5)*P*3 recruits the protein kinase Akt, facilitating its activation by the phosphoinositide-dependent kinase-1, PDK1. Phosphorylation of Akt is critical for the regulation of glucose metabolism, but also for the regulation of cell size, proliferation, and cell survival. In addition, Akt regulates gene transcription by direct phosphorylation of some of the forkhead transcription factors of the FOXO family which are involved in the control of fundamental processes, including cell metabolism and differentiation, apoptosis, cell cycle arrest, and DNA repair [68, 69]. Akt also regulates mRNA translation through the raptor-mTOR pathway, which plays a central role in metabolism and cell growth [70, 71]. The mechanism how activation of the INSR signaling pathway induces growth has been clarified by demonstrating that Akt phosphorylates and inactivates tuberin, an inhibitor of cell growth [72]. It has been shown that activation of PI3K by insulin relieves this inhibitory function [73], resulting in activation of Rheb (Ras homolog enriched in brain), leading to activation of the raptor-mTOR complex. It is well known that PTEN, a lipid phosphatase that dephosphorylates PtdIns(3,4,5)*P*3, negatively regulates the PI3K/Akt signaling pathway, thus emphasizing the role of PTEN as a tumour suppressor in multiple tumour types [74]. In this respect, PTEN is often disrupted in tumour cells, and this emphasizes the role of the insulin/IGF-I-induced PI3K/Akt/mTOR/S6K signaling in cancer [75] (Figure 3).

A second major intracellular signaling pathway involves the Ras protein, a monomeric globular protein of 189 amino acids (21 kDa) which is associated with the plasma membrane and which binds either GDP or GTP. In response to certain growth promoting stimuli, Ras is "switched on" by exchanging its bound GDP for a GTP. Once activated, Ras is able to interact with and activate other downstream protein targets. Switching Ras off requires extrinsic proteins termed GTPase-activating proteins (GAPs) that interact with Ras leading to the conversion of GTP to GDP. Mutations in Ras affecting its ability to interact with GAP, or to convert GTP to GDP, will result in abnormal, prolonged activation of this protein, thus in a sustained signal to the cell that may result in uncontrolled proliferation and disorganized growth of cells. In its active state, Ras binds Raf, a protein kinase, and promotes the activation of a phosphorylation cascade in which a series of serine/threonine protein kinases (the MAP/ERK kinase cascade) are activated in sequence, carrying the signal from the plasma membrane to the nucleus. At the end of this signal cascade, the MAP/ERK-kinase phosphorylates a number of substrates on serines and threonines, including c-Jun, c-Fos, c-Myc, Elk-1, ATF-2, NF-IL6, and TAL-1 p53, thereby modifying their ability to regulate the transcription of genes potentially relevant to cell survival, growth, and cell cycle, such as *Sp1*, *E2F*, *Elk-1,* and *AP-1* [76–79] (Figure 3).

On the whole, dysregulation of the IGF system is well recognized as an important contributor to the progression of multiple cancers, in which constitutive activation of the PI3K/Akt/mTOR signaling and the MAP/ERK-kinase pathway may play a role. Therefore, as underlined elsewhere [80], consistently with these observations, the IGF system is emerging as a promising new target in cancer therapy.

4. Obesity, Diabetes, and Cancer

Many clinical and epidemiological lines of evidence prove that excess body weight gain, associated with hyperinsulinemia, insulin resistance, and dyslipidemia, may be a major

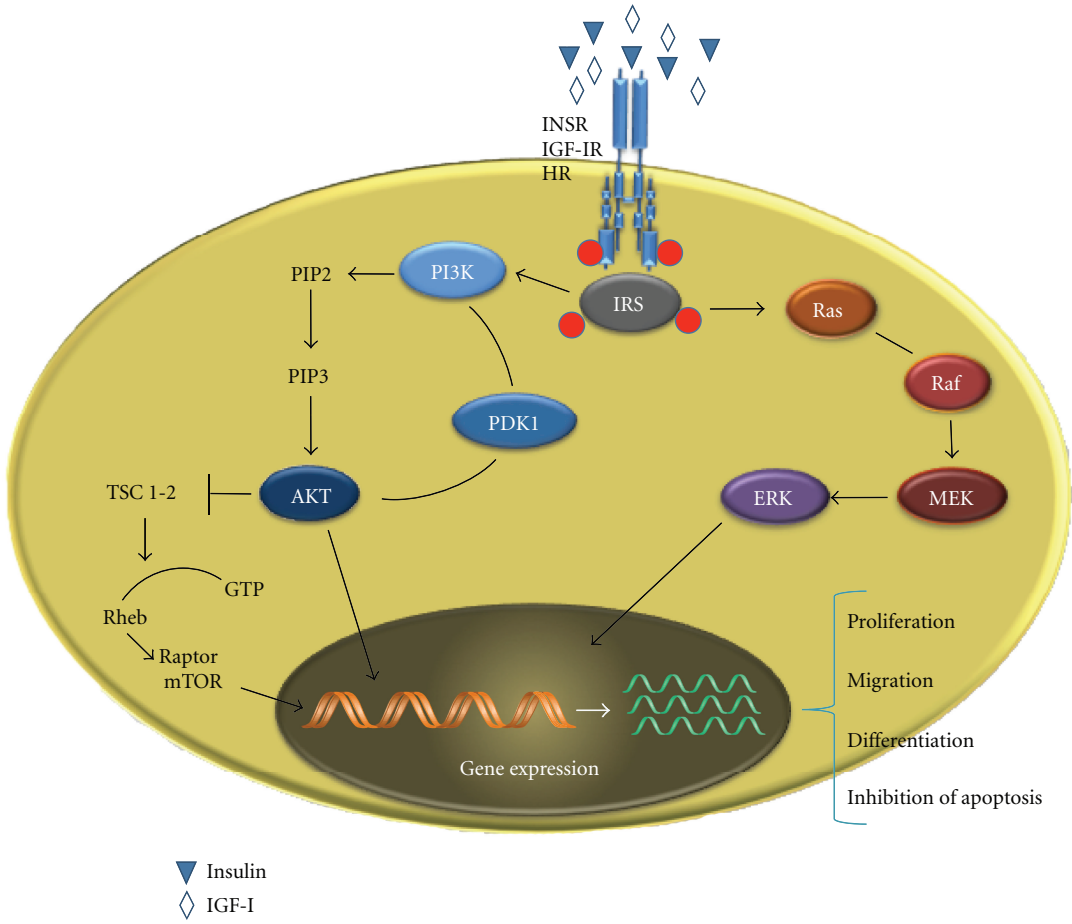

Figure 3: Schematic representation of the two major signaling cascades operating in cancer, following overactivation of the INSR/IGF-IR signaling pathways. Binding of insulin, IGF-I (and IGF-II) triggers the intrinsic tyrosine kinase receptor domain, leading to activation of the PI3K/Akt/mTOR signaling and the MAP/ERK-kinase pathway. HR: hybrid receptors; ERK: extracellular regulated kinase; IRS: INSR substrate; MEK: mitogen-activated protein kinase kinase; mTOR: mammalian target of rapamycin; PI3K: Phosphoinositide-3 kinase; PIP2: phosphatidylinositol [4, 5]-bisphosphate; PIP3: phosphatidylinositol [3, 4, 5]-trisphosphate; PDK1: phosphoinositide-dependent kinase 1; Raf: rapidly fibrosarcoma; Ras: rat sarcoma; Rheb: Ras homolog enriched in brain; TSC: tuberous sclerosis complex.

risk factor for certain types of tumours, including colon and breast cancer (Table 1). As illustrated in Figure 1, in this paper, besides its importance in storage and energy balance, the adipose tissue is metabolically and immunologically active, being able to produce many proteins and hormones known as "adipokines" [97], which include adipocytokines (leptin, adiponectin, and resistin), cytokines (TNF-α, IL-1 and IL-6), and the chemokine MCP-1 [98] that has recently been identified as a potential factor contributing to macrophage infiltration into adipose tissue [99]. Adipokines circulate in the plasma at concentrations that are positively correlated with body mass index (BMI), with the exception of adiponectin, that correlates inversely with BMI [100]. It has been demonstrated that adipocyte-secreted factors can directly promote mammary tumorigenesis through induction of antiapoptotic transcriptional programs and protooncogene stabilization [101]. Also, evidence has been provided indicating that adipocytes in obesity, by the action of adipokines, participate in a highly complex cross-talk with the surrounding tumour cells, promoting tumour progression [102]. Biosynthesis of leptin in adipose tissue is influenced by insulin [103], and this may explain the high leptin levels observed in obesity. Studies have been provided indicating that higher leptin concentrations may constitute a possible link relating obesity and cancer, particularly colorectal cancer. Also, it has been demonstrated that, by influencing specific second intracellular messengers, such as signal transducers and activators of transcription 3 (STAT3), AP-1, ERK2, and MAPK, leptin is involved in breast cancer cell proliferation and survival. On the other hand, greater adiposity in obese or overweight persons down-regulates secretion of adiponectin, an adipokine with anti-inflammatory and insulin-sensitizing properties [104]. Low blood concentrations of adiponectin have been associated with high incidence and poor prognosis of breast cancer, independently from the hormone receptor status [105]. Adiponectin and adiponectin receptors have been found to play a role in the activation of the PPARγ pathway, which, in turn, induces the transcription of several genes involved in the regulation of cell proliferation and differentiation.

Table 1: Relative risk of association between T2D and cancer, as reported by meta-analysis studies.

Cancer	Number (n) of examined studies	Relative risk (CI 95%)	Reference number
Liver	Case control ($n = 13$)	2.50 (1.80–3.50)	[81]
	Cohort ($n = 7$)	2.51 (1.90–3.20)	[81]
	Cohort ($n = 18$)	2.01 (1.61–2.51)	[82]
Endometrium	Case-control ($n = 13$)	2.22 (1.80–2.74)	[83]
	Cohort ($n = 3$)	1.62 (1.21–2.16)	[83]
Pancreas	Case-control ($n = 17$)	1.94 (1.53–2.36)	[84]
	Cohort ($n = 19$)	1.73 (1.59–1.88)	[84]
	Case-control ($n = 3$)	1.80 (1.50–2.10	[85]
	Cohort ($n = 35$)	1.94 (1.66–2.27)	[86]
Kidney	Cohort ($n = 9$)	1.42 (1.06–1.91)	[87]
Biliary tract	Case-control ($n = 8$) and cohort ($n = 13$)	1.43 (1.18–1.72)	[88]
	Case-control ($n = 10$) and cohort ($n = 5$)	1.60 (1.38–1.87)	[89]
Bladder	Case-control ($n = 7$)	1.37 (1.04–1.80)	[90]
	Cohort ($n = 3$)	1.43 (1.18–1.74)	[90]
Colon-rectum	Case-control ($n = 6$)	1.36 (1.23–1.50)	[91]
	Cohort ($n = 9$)	1.29 (1.16–1.43)	[91]
	Case-control + cohort ($n = 14$)	1.38 (1.26–1.51)	[92]
Esophagus	Case-control ($n = 6$) and cohort ($n = 11$)	1.30 (1.12–1.50)	[93]
N-H lymphoma*	Case-control ($n = 5$)	1.12 (0.95–1.31)	[94]
	Cohort ($n = 11$)	1.41 (1.07–1.88)	[94]
	Case-control ($n = 10$)	1.18 (0.99–1.42)	[95]
	Cohort ($n = 3$)	1.79 (1.30–2.47)	[95]
Breast	Case-control ($n = 5$)	1.18 (1.05–1.32)	[96]
	Cohort ($n = 15$)	1.20 (1.11–1.30)	[96]

* *Non-Hodgkin's lymphoma.*

Enhancement of BRCA1 expression by PPARγ has been reported in MCF-7 breast cancer cells [106]. Thus, an explanation for the association of adiponectin with breast cancer is that functional reduction of PPARγ signalling, leading to reduced levels of BRCA1, may impair the DNA repair mechanisms.

Obesity and T2D are frequently associated with increased oxidative stress [107]. However, the functional role of oxidative stress in cancer has long been a hotly debated topic. Recent findings in this context indicate that oxidative stress may directly contribute to tumour progression and metastasis [108]. As recapitulated in Figure 1, one possibility is that ROS overproduction, by triggering the P13K/Akt signaling, could lead to adverse genetic modifications and DNA damage followed by tumour formation and progression [109]. NFκB is a central coordinator of immunity, inflammation, and cell survival. Mutual cross-talk between ROS and NFκB has been identified [110]. For example, fibroblasts harboring activated NFκB are able to promote tumour growth [111]. Activation of NFκB in fibroblasts leads to a loss of Cav-1 which drives onset of "The Reverse Warburg Effect," due to the autophagic destruction of mitochondria (mitophagy) in these cells, resulting in aerobic glycolysis and lactate production [111]. Thus, by using oxidative stress, cancer cells induce the activation of the autophagic program to promote aerobic glycolysis under conditions of normoxia [111]. Therefore, treatment with antioxidants (such as N-acetyl-cysteine, metformin, quercetin, vitamins A, C, and E, selenium and perhaps others) or nitric oxide inhibitors may be beneficial to reverse many of the cancer-associated fibroblast phenotypes [112].

5. Inflammatory Cytokines, Diabetes, and Cancer Risk

Chronic inflammation may represent a link between diabetes and cancer, particularly in the obese, in which visceral fat is infiltrated by macrophages which constitute an important source of proinflammatory mediators [113, 114]. Moreover, macrophage accumulation in adipose tissue is associated with local hypoxia in fat [115]. It has been postulated that hypoxia in the fat tissue of the obese plays a role in the activation of inflammatory macrophages. Colocalization/coordination between macrophages/adipocytes and other cells of the immune system in white fat tissue leads to a low-grade, chronic inflammation that produces many cytokines able to initiate, promote, and sustain tumour progression either directly [116], or indirectly, by causing (via inhibition of the INSR signaling) insulin resistance, which leads to the activation of protumorigenic pathways (see Figure 1). For example, TNF-α, a cytokine involved in systemic inflammation, blocks insulin signaling by preventing serine phosphorylation of IRS-1 [117]. Increased expression of TNF-α has been observed in both acute and chronic inflammatory states, including the chronic inflammatory response associated with cancer, obesity, and diabetes. Overproduction of TNF-α supports and even amplifies the inflammatory process leading to insulin resistance [118].

TNF-α may activate both proapoptotic and antiapoptotic pathways. Under certain circumstances TNF-α may act as a tumour promoter by activating signaling pathways that are critical for life/death decisions, such as MAPKs and the antiapoptotic NFκB pathway. Thus, increased levels of circulating TNF-α may promote tumorigenesis in overweight insulin-resistant patients.

Another well-characterized inflammatory cytokine, IL-6, has also been involved in various metabolic, endocrine, and neoplastic disorders. Activation of STAT signaling, via IL-6, is known to induce cancer cell proliferation, survival, and invasion, while suppressing host antitumour immunity [119]. It has been documented that the expression of IL-6 in adipose tissue and its serum concentrations positively correlate with obesity, insulin resistance, and T2D, even with insulin resistance in cancer patients [97, 120]. In one study with breast cancer patients, IL-6 and estrogen levels were found to be higher in the insulin-resistant breast cancer patients without treatment compared to the ones without insulin resistance [121]. Similarly, in prostate cancer, serum levels of IL-6 were higher in patients with obesity/insulin resistance and clinically evident hormone-resistant prostate cancer, compared to those with hormone-dependent cancer [122]. Inflammation and insulin resistance shift the cell's response to the inflammatory activating NFκB, which is strongly associated with abdominal obesity and insulin resistance. As stated above, this transcription factor is involved in cytokine signaling and in cell survival, and its expression is induced by a multitude of different extracellular stimuli, including chemotherapeutics, stress stimuli, and growth factors. NFκB promotes the expression of target genes involved in cellular proliferation and cell migration, anti-apoptosis, and angiogenesis. Functional reduction of NFκB correlates with decreased breast tumour cell proliferation. Another mechanism that fuel cancer growth and tumour progression in low-grade chronic inflammation and insulin resistance is the accumulation of damaged DNA [123, 124]. Hyperglycemia in insulin resistance increases advanced glycation end-product (AGE) formation [125]. The production of intracellular AGE precursors damages target cells by modifying proteins and altering their function. It has been reported that plasma proteins modified by AGE precursors bind to AGE receptors on endothelial and mesangial cells and macrophages, inducing receptor-mediated production of ROS. Also, AGE receptor ligation, by activating NFκB, can induce adverse changes in gene expression [126].

6. Conclusions

The last decades of medical research examining the pathogenesis of common tumours have provided compelling evidence for the involvement of insulin resistance in cancer. Consequently, many research articles have been published in the literature which give support to the hypothesis that patients with insulin-resistant syndromes, such as obesity and T2D, might be at higher risk for developing cancer than the general population. The molecular mechanisms for this association are unknown, but chronic sustained hyperinsulinaemia in these insulin-resistant patients appears to play a role in the neoplastic transformation process. As underlined in this paper, several explanations have been proposed for this association; however the precise mechanisms that link insulin resistance and cancer have not yet been fully understood and a more detailed molecular and mechanistic understanding is required to interpret the existing data, together with more thorough preclinical and clinical studies. Understanding these mechanisms may lead to novel diagnostic and therapeutic strategies in these patients in which measures to decrease chronic hyperinsulinemia and insulin resistance may offer a general approach to prevention of cancer.

Abbreviations

AGE: Advanced glycation end-product
AP2-α: Activator protein 2-alpha
ATF-2: Activating transcription factor-2
C/EBPβ: CCAAT/enhancer binding protein beta
Cav-1: Caveolin-1
ERK: Extracellular regulated kinase
GAP: GTPase-activating protein
HMGA1: High-mobility group A1
IGF-I: Insulin-like growth factor-I
IGF-IR: IGF-I receptor
IGFBP: Insulin-like growth factor binding protein
INSR: Insulin receptor
IRS-1: Insulin receptor substrate-1
MAP: Mitogenic activated protein
MCP-1: Monocyte chemoattractant protein-1
MEK: Mitogen-activated protein kinase kinase
mTOR: Mammalian target of rapamycin
NFκB: Nuclear factor kappa B
PDK1: Phosphoinositide-dependent kinase 1
PI3K: Phosphoinositide-3 kinase
PPAR: Peroxisome proliferator-activated receptor
PTEN: Phosphatase and tensin homolog
Raf: Rapidly fibrosarcoma
Ras: Rat sarcoma
Rheb: Ras homolog enriched in brain
ROS: Reactive oxygen species
SHBG: Sex-hormone-binding globulin
Sp1: Specificity protein 1 transcription factor
STAT: Signal transducer and activator of transcription
T2D: Type 2 diabetes mellitus
TAL-1: T-cell acute lymphocytic leukemia protein-1
TNF-α: Tumour necrosis factor-alpha
TSC: Tuberous sclerosis complex.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

A Review on the Association between Glucagon-Like Peptide-1 Receptor Agonists and Thyroid Cancer

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There is a concern on the risk of thyroid cancer associated with glucagon-like peptide-1 (GLP-1) analogs including liraglutide and exenatide. In this article, we review related experimental studies, clinical trials and observational human studies currently available. In rodents, liraglutide activated the GLP-1 receptors on C-cells, causing an increased incidence of C-cell neoplasia. Animal experiments with monkeys demonstrated no increase in calcitonin release and no C-cell proliferation after long-term liraglutide administration. Longitudinal 2-year data from clinical trials do not support any significant risk for the activation or growth of C-cell cancer in humans in response to liraglutide. However, an analysis of the FDA adverse event reporting system database suggested an increased risk for thyroid cancer associated with exenatide after its marketing. Noticeably, a recent study discovered that GLP-1 receptor could also be expressed in human papillary thyroid carcinomas (PTC), but the impact of GLP-1 analogs on PTC is not known. Therefore, GLP-1 analogs might increase the risk of thyroid C-cell pathology in rodents, but its risk in humans awaits confirmation. Since GLP-1 receptor is also expressed in PTC besides C-cells, it is important to investigate the actions of GLP-1 on different subtypes of thyroid cancer in the future.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin hormone released after meals by L cells in the ileum [1]. It increases the secretion of insulin from the pancreas in a glucose-dependent manner and suppresses the secretion of glucagon, a counter-hormone to insulin [2]. There are two GLP-1-mimetic drugs currently approved for clinical use to treat type-2 diabetes, that is, exenatide and liraglutide [3, 4]. Exenatide is the first GLP-1 receptor agonist approved in April 2005 for the treatment of type-2 diabetes mellitus. It is a 39-amino acid peptide with 53% amino acid homology to full-length GLP-1 [4]. With elimination by glomerular filtration [5] and a mean half-life of 3.3–4 hours [6], exenatide has to be injected subcutaneous twice a day. On January 25, 2010, the FDA approved liraglutide, a GLP-1 receptor agonist that can be injected once daily to improve glycemic control in adults with type-2 diabetes [3, 4]. Liraglutide is a long-acting GLP-1 analog with one amino acid substitution (Arg34Lys) and an attachment of a C-16-free-fatty acid derivative via a glutamol spacer to Lys26 [4]. These modifications lead to slower absorption rate from injection site, higher binding affinity to albumin, and a plasma half-life of 11–13 hours [7–9]. While GLP-1 analogs can efficiently reduce blood glucose level in patients with type-2 diabetes [3, 4], they may potentially have adverse effects on thyroid glands because GLP-1 receptors are expressed in thyroid glands of humans [10] as well as in those of rodents [11]. In preclinical animal studies, rodents treated with liraglutide would have a higher incidence of C-cell tumor formation and focal hyperplasia [12, 13]. It is possible that long-term exposure to GLP-1 receptor agonists in humans may also induce C-cell neoplasia since GLP-1 receptors are expressed in the human thyroid glands [10].

Both the prevalence and incidence of diabetes have been increasing dramatically in recent decades, especially in the Asian people [14]. Diabetes is also one of the leading causes of death nowadays [15]. The link between diabetes and

cancers has become a great concern recently, and the use of antidiabetic drugs may partially contribute to such an increased cancer risk in the diabetic patients [16–25]. For examples some clinical trials have suggested an association between pioglitazone and bladder cancer [26, 27]. In this paper, we reviewed experimental studies, controlled clinical trials, and observational human studies currently available on the association between GLP-1 analogs and thyroid cancer.

2. Experimental *In Vitro* and Animal Studies in Rodents

Calcitonin, a hormone secreted by thyroid C cells, is regarded as an important clinical biomarker for C-cell diseases such as medullary thyroid carcinoma (MTC) and hereditary C-cell hyperplasia because of its high sensitivity and specificity [28–30]. Several *in vitro* studies employing rat thyroid C-cell lines and thyroid tissues have demonstrated that activation of the GLP-1 receptor leads to calcitonin secretion, which is attenuated by the GLP-1 receptor antagonist exendin (9–39) [31, 32]. The functional effect of GLP-1 receptor agonists on rat C-cell lines was investigated by Knudsen et al. [11]. They found that GLP-1 receptor agonists elicited calcitonin release and calcitonin gene expression in a dose-dependent manner in rodent C cells. GLP-1 receptor agonists, including native GLP-1, exenatide, and liraglutide, activated rodent thyroid C cells to release calcitonin in a GLP-1 receptor-dependent manner.

In addition to the *in vitro* studies, Knudsen et al. designed animal experiments to study the development of C-cell hyperplasia and tumor formation after long-term dosing with GLP-1 receptor agonists in rodents [11]. The incidences of both C-cell hyperplasia and C-cell tumor formation at 104 weeks increased in a dose-dependent manner and reached statistical significance. The minimum doses of liraglutide to cause a statistically significant increase in the incidence of C-cell hyperplasia were 0.25 mg/kg/day in rats and 0.2 mg/kg/day in mice. Both doses were 2-fold greater than the equivalent human dose of 1.8 mg/day [11, 12]. The minimum doses to induce a significant increase in C-cell tumor formation in rats and mice were 0.075 mg/kg/day and 1.0 mg/kg/day, respectively; the dose in rats (0.075 mg/kg/day) was equivalent to the dose recommended for treatment of type-2 diabetes in humans [11, 12]. On the other hand, C-cell tumors occurred in mice receiving a daily dose of liraglutide that was 10-fold greater than the corresponding human dose [11, 12]. Of note, in 2-year studies involving wild-type mice, dose-dependent C-cell hyperplasia and neoplasia developed only at doses that also caused increased calcitonin levels [11]. Together, *in vitro* and *in vivo* experiments have demonstrated that long-term GLP-1 receptor activation is associated with increased calcitonin gene transcription and subsequently with C-cell proliferation and tumor formation in both rats and mice.

Madsen et al. [33] documented that C-cell hyperplasia and calcitonin release associated with GLP-1 agonists in wild-type mice were GLP-1-receptor dependent. Besides, C-cell effects seen in mice were not associated with the activation of the rearranged-during-transfection (RET) protooncogene. GLP-1 activates the mammalian target of rapamycin (mTOR) pathway by stimulating the production of cAMP. Activation of mTOR in turn results in downstream phosphorylation of ribosome S6. In contrast, these effects were not observed in GLP-1-receptor knock-out mice.

3. Experimental *In Vitro* and Animal Studies in Primates

In addition to their experiments in rodents, Knudsen et al. [11] investigated the effect of GLP-1 receptor agonists on human TT C cells and on nonhuman primates (cynomolgus monkeys). In contrast to the results in rodents, GLP-1 receptor agonists did not stimulate calcitonin release in human TT C cells. The expression of GLP-1 receptor in human TT C cells was very low, and the corresponding mRNA transcripts were 14- to 21-fold lower than in rat C cells. Furthermore, *in vivo* animal studies with cynomolgus monkeys demonstrated no calcitonin release and no effects on the relative C-cell fraction in the thyroid gland or C-cell proliferation in monkeys after up to 87 weeks dosing with liraglutide at 5.0 mg/kg/day. This liraglutide dose was up to 60-fold greater than the highest dose recommended for the treatment of type-2 diabetes.

4. Human Clinical Trials and Epidemiologic Studies

Additionally, a series of clinical trials in over 5,000 patients with either diabetes or nondiabetic obesity was presented by Hegedüs et al. [34]. In all trials, subjects were randomized to receive liraglutide at doses ranging from 0.6 to 3.0 mg, active comparator, and/or placebo. Calcitonin concentration was monitored at baseline and at 12-week intervals, thereafter, in all subjects enrolled in 8 phase-3 clinical trials (the liraglutide effect and action in diabetes (LEAD) trials 1–6, and 2 phase-3 trials in Japanese subjects) and in 1 phase-2 trial with nondiabetic obese subjects. These trials had intervention phases of 20 to 104 weeks. When combining data from these 9 clinical studies, there was no consistent change in calcitonin levels over time (up to 104 weeks) with any dose of liraglutide or between treatment groups in the proportion of patients whose calcitonin levels increased above a clinically relevant cut-off value of 20 pg/mL. The proportion of subjects switching to a higher calcitonin category, defined by the upper normal range (UNR; <UNR, UNR-2UNR, ≥2UNR) or by potential calcitonin signals of 20, 50, and 100 ng/L for C-cell abnormalities (<20 ng/L, 20 to <50 ng/L, 50 to <100 ng/L, and ≥100 ng/L), was low and did not differ between the liraglutide and active comparator groups. In the LEAD-6 trial [3], the calcitonin responses to distinct GLP-1 receptor agonists (liragutide 1.8 mg once daily versus exenatide 10 μg twice daily) were compared. No difference was seen in estimated geometric mean calcitonin over 26 weeks between the 2 groups. In the trial with nondiabetic obese subjects, treatment groups receiving

Table 1: *In vitro*/*in vivo* experiments and clinical studies on the association between GLP-1 analogs and C-cell pathology.

Authors [reference]	Studied drugs	Research materials or study subjects	Main outcomes investigated	Main findings
		Cellular models		
Crespel et al. [32]	Glucagon, GLP-1 (7–36), and exendin (9–39)	Rat CA-77 C-cell line	cAMP production and calcitonin secretion	GLP-1 (7–36) and glucagon dose dependently stimulated cAMP production and calcitonin secretion. Exendin (9–39) abolished a further increase in cAMP formation at glucagon concentration over 10 nM and partially suppressed glucagon-induced calcitonin secretion.
Lamari et al. [31]	GLP-1 (7–37), and exendin (9–39)	Rat CA-77 C-cell line	cAMP production, calcitonin mRNA levels, and calcitonin secretion	GLP-1 (7–37) increased cAMP formation in a dose-dependent manner. Exedin (9–39), an antagonist of GLP-1 receptor, blunted the stimulation of cAMP production induced by GLP-1 (7–37). Gene expression and peptide secretion of calcitonin were increased after incubation of CA-77 cells with GLP-1 (7–37).
Knudsen et al. [11]	Liraglutide, exenatide, and GLP-1 (7–37)	Human TT C-cell line, rat MTC 6–23 C-cell line, and rat CA-77 C-cell line	GLP-1 receptor mRNA and protein expression; calcitonin release after GLP-1 receptor agonists	Native GLP-1, liraglutide, and exenatide all stimulate calcitonin gene expression and calcitonin secretion via the GLP-1 receptor in a dose-dependent manner in rat C cells. The human TT cells express few GLP-1 receptors compared with rat MTC 6–23 and CA-77 and show a lack of functional response to GLP-1 and GLP-1 receptor agonists.
		Animal experiments		
(I) Rodents				
Knudsen et al. [11]	Liraglutide versus vehicle control	Sprague Dawley rats aged 6-7 weeks and CD-1 mice at the age of 5–10 weeks	Plasma calcitonin and pathological examination to thyroid gland sections after dosing with liraglutide	Calcitonin levels increase with time and dose with 104-week repeated dosing of liraglutide. The incidences of both C-cell hyperplasia and C-cell tumor formation at 104 weeks were increased in a dose-dependent manner and reached statistical significance.
Madsen et al. [33]	Liraglutide, exenatide, and vehicle control	CD-1 wild-type mice aged 5-6 weeks and GLP-1-receptor knockout mice at the age of 4-5 weeks	Plasma calcitonin, pathological examination to thyroid tissue sections, and immunohistochemical staining for phosphoproteins after 13-week treatment with liraglutide or exenatide	GLP-1 agonists cause calcitonin release and C-cell hyperplasia in wild-type mice via a GLP-1-receptor-dependent mechanism. GLP-1 activates the mammalian target of rapamycin (mTOR) pathway by stimulating the production of cAMP. Activation of mTOR in turn results in downstream phosphorylation of ribosome S6. Liraglutide-induced C-cell hyperplasia in mice is not associated with RET activation.

Table 1: Continued.

Authors [reference]	Studied drugs	Research materials or study subjects	Main outcomes investigated	Main findings
(II) Primates				
Knudsen et al. [11]	Liraglutide versus vehicle control	Cynomolgus monkeys aged 1-2 years	Plasma calcitonin and pathological analysis to thyroid gland sections after dosing with liraglutide	No increase in plasma calcitonin was seen in cynomolgus monkeys receiving a single dose of liraglutide or during 87-week daily dose. There was also no change in the thyroid gland sections, relative C-cell fraction of the thyroid gland, and proliferative index in the C cells after liraglutide for 52 weeks.
			Human studies	
Gier et al. [10]	—	Human thyroid glands	Expression of GLP-1 receptors in tissue samples with C-cell abnormalities, papillary thyroid cancer, and normal thyroid	GLP-1 receptor immunoreactivities were detected in 33%, >90%, and 18% of patients with normal C cells, C-cell pathologies, and PTC lesions, respectively.
Hegedüs et al. [34]	Liraglutide versus active comparators and placebo	Nine clinical trials of 20–104-week duration	Geometric mean levels of serum calcitonin and outlier analysis	There was no significant difference in mean calcitonin levels between liraglutide and control groups. The proportions of subjects with calcitonin levels shifting to a higher category or above a clinically relevant cut-off value of 20 pg/mL were low and did not differ between treatment groups.
Elashoff et al. [36]	Exenatide versus rosiglitazone	Adverse effect reporting system	Overall thyroid cancer	Odds ratio for thyroid cancer was 4.73; $P = 4 \times 10^{-3}$

a higher liraglutide dose (1.2–3 mg/day) were compared with those receiving orlistat (360 mg/day) or placebo. The estimated geometric mean calcitonin levels over 52 weeks remained at the low end of the normal range in all treatment groups. Regarding clinical adverse events related to C cells, 6 subjects were found to have histologically documented C-cell hyperplasia with identical prevalence in the liraglutide and nonliraglutide groups. One case of MTC was described in a subject who was not treated with liraglutide. Although there were fluctuations in calcitonin levels among these 7 subjects during the trial periods, no consistent pattern was discovered. Taking together, these data do not support any significant risk for the activation or growth of C cells in humans in response to GLP-1 receptor agonists over the 2-year period.

Although GLP-1 receptor stimulation induced calcitonin release and C-cell proliferation in rodents, these effects were not observed in primates [11], implying possible species-specific differences in GLP-1 receptor expression and activation in the thyroid. Computer-assisted cell counting in sections stained immunohistochemically for calcitonin revealed that the C-cell densities in thyroid glands from cynomolgus monkeys and humans were comparable, and more importantly, that the C-cell densities in thyroid glands in mice and rats were 22- and 45-fold higher, respectively, than that in humans [11].

5. GLP-1 Receptor Expression in Thyroid Tumors Derived from Follicular Cells and Its Functional Significance

Recently, Gier et al. [10] examined thyroid tissue samples procured at surgery from individuals with C-cell hyperplasia and those with MTC for the presence of GLP-1 receptor expression using immunocytochemical techniques. C-cells within relatively normal tissue without any hyperplasia or neoplastic changes were also evaluated for GLP-1 receptor expression. In this study, calcitonin-expressing C cells were immunoreactive for GLP-1 receptor in 33% (5/15), 91% (10/11), and 100% (9/9) of individuals with normal thyroid lobes, MTC, and C-cell hyperplasia, respectively. There was no immunoreactivity for either calcitonin or the GLP-1 receptor in normal thyroid follicles identified in the same sections. Furthermore, there was no correlation between the extent of GLP-1 receptor immunoreactivity and either tumor size or plasma calcitonin concentrations. Moreover, the expression of calcitonin and GLP-1 receptors was investigated in thyroid tissues obtained from 17 individuals with papillary thyroid cancer (PTC). PTC cells were negative for calcitonin. But GLP-1 receptor immunoreactivity was unexpectedly present in PTC cells in 3 of the 17 (18%) cases. In other words, the GLP-1 receptor is not expressed in normal thyroid follicular cells but may be aberrantly present in a subset of PTCs derived from follicular cells. The functional significance of these findings has not yet been ascertained. PTC is the most common malignancy of the thyroid, constituting approximately 50–90% of thyroid malignancies worldwide [35]. Thus, GLP-1 receptor immunopositivity in a subset of PTC lesions is likely to be of greater epidemiological significance than in C-cell neoplasms. Elashoff et al. [36] examined the FDA adverse event reporting system (AERS) database for thyroid cancer in association with exenatide, another human GLP-1 analog. The reported event rate for thyroid cancer was 4.73-fold greater in patients treated with exenatide compared to the control drug, rosiglitazone ($P = 4 \times 10^{-3}$). Although it is impossible to know the thyroid cancer subtypes reported in FDA AERS database, these findings indicate that it is important to carefully monitor individuals exposed to long-term pharmacological GLP-1 analogs for any increased incidence of thyroid malignancies. In addition, more detailed future investigation into the actions of GLP-1 on each subtype of thyroid cancers, especially PTC, is required.

6. Conclusion

Table 1 summarizes the findings on thyroid cancers after dosing with GLP-1 analogs from experimental studies, controlled clinical trials and observational studies (Table 1). Data from studies in rodents suggested that GLP-1 analogs were associated with an increased risk of thyroid C-cell hyperplasia and C-cell tumors. On the other hand, animal experiments with monkeys did not show increased calcitonin release or proliferation of C-cells in thyroid glands after chronic administration of liraglutide. Longitudinal data from clinical trials have not demonstrated a causal association between GLP-1 analogs and thyroid C-cell pathology over a 2-year period. However, long-term observational studies are required to monitor such a potential risk in human. It is worth noting that a subset of human PTC expresses GLP-1 receptors. The FDA AERS database supported an increased risk of thyroid cancer associated with exenatide. It is urgent to investigate the actions of GLP-1 on each subtype of thyroid cancers, especially PTC, in the future. Besides, caution is warranted in the use of this class of agents, especially in individuals with a history of thyroid cancer.

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