Liver paraoxonase 3 expression and the effect of liraglutide treatment in a rat model of diabetes

Yuntao Liu1.B.C, Dan Zhu2.A,F, Guofeng Dong1.C.D, Yuqin Zeng3.C,D, Pan Jiang1.D,E, Yaoling Xiao4.F

1 Department of Endocrinology, Affiliated Ren He Hospital of China Three Gorges University, Second Clinical Medical College of China, Three Gorges University, Yichang, China
2 Third Clinical Medical College of China Three Gorges University, Gezhouba Central Hospital of Sinopharm, Yichang, China
3 Hemodialysis room, Affiliated Ren He Hospital of China Three Gorges University, Second Clinical Medical College of China Three Gorges University, Yichang, China
4 Department of Endocrinology, Third Clinical Medical College of China Three Gorges University, Gezhouba Central Hospital of Sinopharm, Yichang, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Address for correspondence
Dan Zhu
E-mail: danzhudr@tom.com

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Abstract

Background. This study investigated liver expression of paraoxonase 3 (PON3), phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and nuclear factor (NF)-κB in a rat model of type-2 diabetes mellitus (T2DM), and assessed the effect of liraglutide treatment.

Objectives. To investigate liver PON3 expression in rats with T2DM assess its role in disease pathogenesis, and determine the effect of liraglutide on its expression.

Material and methods. Type 2 diabetes mellitus was induced in 3 groups of rats: positive control group (PC; no treatment), and low-dose (LL; 100 μg/kg) and high-dose (HL; 200 μg/kg) liraglutide groups. Healthy rats served as a normal control (NC) group. Protein and mRNA expression were measured with western blot and reverse-transcriptase polymerase chain reaction (RT-PCR), respectively.

Results. After liraglutide treatment, fasting plasma glucose (FPG), homeostasis model assessment-insulin resistance (HOMA-IR), fasting insulin (FINS), malondialdehyde (MDA), and interleukin 6 (IL-6) levels were lower in HL rats compared with LL ones (p < 0.05). Compared to NC rats, FPG, FINS, HOMA-IR, low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), and IL-6 levels were the lowest in HL rats, followed by LL and PC ones (p < 0.05). Body weight (BW) was lower in LL and HL groups than in NC and PC (p < 0.05). The liver expression of PON3, PI3K and Akt were the highest in HL rats, followed by LL and PC (p < 0.05). The NF-κB expression was the lowest in HL rats, followed by LL and PC (p < 0.05). The PON3 expression was decreased in the diabetic rat liver.

Conclusions. Liraglutide can increase PI3K, Akt and PON3 expression, and decrease NF-κB expression. The effect of liraglutide on improving insulin resistance and abnormal glucolipid metabolism in T2DM rats may be associated with increased liver PON3 expression.

Key words: type 2 diabetes mellitus, liraglutide, paraoxonase 3, proprotein convertase subtilisin 9
Background

Paraoxonase 3 (PON3), a newly discovered member of the paraoxonase family, is closely related to insulin resistance, lipid metabolism and obesity.1,2 It has an inhibitory effect on inflammatory factors and can improve insulin resistance.3 Phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) play a role in regulating PON3 expression.4

As a long-acting analog of glucagon-like peptide-1 (GLP-1), liraglutide has the same physiological effects as natural GLP-1 through molecular modification, and these are persistent due to its long half-life low decomposition rate. Liraglutide has the same physiological effects as natural GLP-1 through molecular modification, and these are persistent due to its long half-life low decomposition rate. Liraglutide is used as a potent hypoglycemic agent in clinical practice, and has been shown to reduce body mass index (BMI) and improve insulin resistance.5 Moreover, it can significantly improve dysfunctional glycolipid metabolism in metabolic diseases and reduce diabetes complications. It has also been found to increase PI3K and Akt expression and inhibit inflammation.6–8

Objectives

The aim of this study was to investigate liver PON3 expression in rats with type 2 diabetes mellitus (T2DM), assess its role in disease pathogenesis and determine the effect of liraglutide on its expression.

Material and methods

Materials

Test materials were: streptozotocin (STZ) (Sigma-Aldrich, St. Louis, USA); peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), irisin primers, and glyceraldehyde 3-phosphate dehydrogenase (GADPH) primers (Boster, Wuhan, China); primary antibodies (Protein tech, Rosemont, USA); and secondary antibodies (Boster). A reverse transcription polymerase chain reaction (RT-PCR) kit was purchased from Invitrogen (Carlsbad, USA).

Methods

Forty-four four-week-old, specific pathogen-free, male Sprague–Dawley rats weighing 190–210 g were purchased from the Experimental Animal Center of Wuhan University, China (animal certificate No. SCXK (E) 2017-0012). All rats were kept in specific pathogen-free rooms controlled for temperature (22–26°C) and humidity (40–70%), with 12-hour light/dark light cycle and free access to food and drinking water except during the experiments. Following 7 days of acclimatization, rats were randomly divided into a normal control (NC) group (n = 10) and experimental group (n = 34). The NC rats were fed a normal diet, and experimental rats received a high-fat diet for 2 weeks. Subsequently, T2DM was induced in the experimental rats by ip. administration of STZ (40 mg/kg body weight (BW) Seventy-two hours after STZ administration a total of 30 out of 34 rats had glucose levels ≥16.7 mmol/L and were considered diabetic. Diabetic rats were fed a high-fat diet for another 2 weeks and randomly divided into 3 treatment groups (n = 10 each): positive control (PC; no treatment) group, and low-dose liraglutide (LL) and high-dose liraglutide (HL) groups. The LL and HL rats were given intraperitoneal injections of liraglutide 100 μg/kg/day and 200 μg/kg/day, respectively, for 4 weeks. The NC and PC rats were ip. injected with the same volume of normal saline for 4 weeks.

Sample collection

Tail vein blood was collected from each of the control and diabetic rats and stored at −80°C for later use after T2DM was confirmed. At the end of week 17, tail vein blood was collected again from each of the control and diabetic rats fasted for 12 h and anesthetized with pentobarbital. Liver tissue was promptly excised from anesthetized rats and washed with normal saline (NaCl 0.9%) after blood collection. The harvested tissues were then dried using filter paper and stored at −80°C. Fasting plasma glucose (FPG) was measured using a glucometer (Johnson & Johnson, New Brunswick, USA). Plasma levels of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglyceride (TG) were measured using an Abbott C8000 automatic analyzer (Abbott Laboratories, Chicago, USA). Interleukin 6 (IL-6) was measured with an enzyme-linked immunosorbent assay (ELISA; Boster) using a Bioteck ELx800 microplate reader (BioTek, Winooski, USA). Insulin was determined using electrochemiluminescence (Cobas e411 autoanalyzer; Roche, Basel, Switzerland). Malondialdehyde (MDA) and protein kinase B (Akt) were measured using an Abbott C8000 automatic analyzer (Abbott Laboratories, Chicago, USA). Interleukin 6 (IL-6) was measured with an enzyme-linked immunosorbent assay (ELISA; Boster) using a Bioteck ELx800 microplate reader (BioTek, Winooski, USA). Insulin was determined using electrochemiluminescence (Cobas e411 autoanalyzer; Roche, Basel, Switzerland). Malondialdehyde (MDA) assay kits were purchased from Jiancheng (Nanjing, China). Fasting insulin (FINS) was assessed with a double antibody radioimmunoassay. Homeostasis model assessment-insulin resistance (HOMA-IR) was computed as FBG × FIN/22.5.

Liraglutide (3 mL) was purchased from Novo Nordisk ( Bagsværd, Denmark). The basal diet was composed of sorghum flour, soy flour, corn flour, cod liver oil, wheat bran, multivitamins, and trace elements, with a fat content of 4% (HFK Bioscience, Bagsværd, Denmark). The high-fat feed contained lard oil, cholesterol, egg yolk powder, and deoxycholate in addition to the basal diet (carbohydrate 30.01%, protein 16.24%, fat 53.75%, total calories 486 kcal/100 g; Boster).

Determination of liver PON3, PI3K, Akt, and NF-κB mRNA expression using RT-PCR

PON3, PI3K/Akt and NF-κB mRNA expression were determined using a nucleic acid/protein analyzer. For RNA extraction, 1 mL of TRIzol reagent was added to about 100 mg of fresh frozen liver tissue according
to the single-step TRIzol method. The RNA concentration and purity were determined. The cDNA was synthesized from the same amount of RNA. An appropriate amount of cDNA was then amplified using PCR.

**Determination of PON3, Akt and NF-κB protein expression using western blot**

A small amount of liver tissue was placed in a 1-mL or 2-mL homogenizer, minced with scissors, mixed with 400 μL of single-detergent lysis buffer (containing phenylmethylsulfonyl fluoride (PMSF)), homogenized, and placed on ice. After a few minutes, the tissue was ground and then placed on ice again. This step was repeated several times to obtain the desired fineness. After lysis for 30 min, the lysate was transferred to a 1.5-mL centrifuge tube with a pipette and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was aliquoted into 0.5-mL centrifuge tubes and stored at −20°C. Signals generated by horseradish peroxidase (HRP)-conjugated antibodies were visualized using an enhanced chemiluminescence detection system and exposed on Kodak X-ray film (Eastman Kodak, Rocherster, USA). Protein band intensities were quantified with densitometric analysis using BandScan v. 4.3 software (Glyko, Shanghai, China) and corrected to GAPDH levels.

**Statistical analysis**

Statistical analysis was performed using SPSS v. 17.0 software (SPSS Inc., Chicago, USA). All data was expressed as X ± s. Differences between groups were analyzed using one-way analysis of variance (ANOVA). The least significant difference (LSD) t-test was used for comparisons between the 2 groups. For all tests, p < 0.05 was considered statistically significant.

**Results**

Figure 1 shows the relationship between FBG, HOMA-IR and FINS levels before and after liraglutide treatment. Significantly higher FBG, HOMA-IR and FINS levels were found in PC, LL and HL rats compared to NC rats.

![Fig. 1. FBG, HOMA-IR and FINS levels before and after liraglutide treatment](image1)

**Fig. 1. FBG, HOMA-IR and FINS levels before and after liraglutide treatment**

# p < 0.05 compared to NC rats before treatment; △ p < 0.05 compared to NC rats after treatment; ▲ p < 0.05 compared to PC rats after treatment; ★ p < 0.05 before and after treatment.

![Fig. 2. LDL-C, TG, TC and HDL-C levels, and BW before and after liraglutide treatment](image2)

**Fig. 2. LDL-C, TG, TC and HDL-C levels, and BW before and after liraglutide treatment**

# p < 0.05 compared to NC rats before treatment; △ p < 0.05 compared to NC rats after treatment; ▲ p < 0.05 compared to PC rats after treatment; ★ p < 0.05 before and after treatment.
(p < 0.05) before treatment. However, after treatment, these levels were significantly lower in HL rats than in LL rats (p < 0.05).

Figure 2 shows LDL-C, TG, TC, and HDL-C levels and BW before and after liraglutide treatment. A significantly higher TG level and BW were found in PC, LL and HL rats compared to NC rats (p < 0.05) before treatment. Nevertheless, BW was lower in LL and HL rats than in NC and PC rats after treatment, but no significant higher TC levels was found in HL rats than in LL rats (p > 0.05).

Figure 3 presents IL-6 and MDA levels before and after liraglutide treatment. Significantly higher IL-6 and MDA levels were detected in PC, LL and HL rats compared to NC rats (p < 0.05) before treatment, yet significantly lower in HL rats than in LL rats (p < 0.05) after treatment. Compared to NC rats, FPG, FINS, HOMA-IR, LDL-C, TG, and IL-6 levels were the lowest in HL rats, followed by LL and PC rats (p < 0.05).

Figures 4–6 depict liver PI3K, Akt protein, liver PON3 protein, liver NF-κB protein, and mRNA expression in each
group. The results showed that liraglutide increased PI3K, Akt and PON3 expression in LL and HL rats. However, it decreased FBG and HOMA-IR levels.

Discussion

The PON3 expression is closely related to insulin resistance, lipid metabolism and obesity.1,2 In this study, liver PON3 expression was decreased in PC rats, which further suggests that low PON3 levels may be involved in the pathogenesis of T2DM. Moreover, PI3K and Akt expression in liver were increased after liraglutide treatment, accompanied by increased PON3 levels. A recent study found that PI3K and Akt enhanced PON3 expression.4 Another study showed that liraglutide increased the expression of PI3K and Akt,6 which is supported by our results. Therefore, it is possible that liraglutide stimulated PON3 expression by upregulating PI3K and Akt expression. In this study, PON3 expression was increased in LL and HL rats, accompanied by lowered FBG and HOMA-IR levels. Liraglutide might improve insulin resistance and glucose metabolism by upregulating PON3 expression.

Inflammation is involved in the pathogenesis and progression of T2DM. We also found higher serum IL-6 levels in PC rats compared to NC rats. Beek et al. found that patients with T2DM had higher serum IL-6 levels than normal subjects.7 It has also been reported that high IL-6 levels inhibited insulin secretion10 and produced cytotoxic effects that caused pancreatic β cell death.11 Interleukin 6 can also induce T2DM by reducing cell-surface glucose transporter 4 expression.12 This study also found higher IL-6 levels in PC rats, suggesting that increased IL-6 levels might be involved in T2DM pathogenesis. Liraglutide has been shown to inhibit inflammation,7 which is supported by our observation of decreased IL-6 levels in both LL and HL rats. Moreover, liraglutide-treated rats showed higher PON3 expression, along with lower IL-6 levels and NF-κB expression, as compared to PC rats. The PON3 overexpression inhibits inflammatory factors in CCl₄-induced liver injury. Schweikert et al.13 suggested that PON3 reduced the activation of the NF-κB inflammatory pathway, thereby inhibiting inflammation. Thus, it can be seen that inhibition of the NF-κB inflammatory pathway is one of the anti-inflammatory mechanisms of PON3. It is possible that the effect of liraglutide on IL-6 levels is partially achieved by upregulating PON3 expression and thereby inhibiting NF-κB. The lower IL-6 levels in HL rats than in LL rats may be associated with the greater increase of PON3 expression in HL rats. The PON3 expression was decreased in PC rats. It is possible that the downregulation of PON3 may reduce the inhibition of inflammation, thereby promoting the pathogenesis of T2DM.

Furthermore, NF-κB expression was increased in PC rats, accompanied by higher MDA levels. The NF-κB can promote the production of reactive oxygen species (ROS).14,15 The ROS-mediated oxidative stress can cause damage and dysfunction of insulin target organs and tissues, and is closely related to T2DM and obesity. Reactive oxygen species have also been shown to activate NF-κB signaling,16,17 suggesting that the interaction between ROS and NF-κB further provokes oxidative stress. Bharathidevi et al. found that PON3 played a defensive role against oxidative stress in diabetic retina.18 Ferreira et al. reported that PON3 was expressed in bull testis and prevented oxidative damage associated with male reproductive function.19 The abovementioned studies demonstrate that PON3 protects against oxidative stress. It has been suggested that the antioxidative stress effect of PON3 may be related to NF-κB inhibition.20 It is possible that reduced PON3 expression in PC rats was also involved in increased oxidative stress by reducing NF-κB inhibition. Liraglutide protects against oxidative stress.20 In this study, PON3 expression was increased and NF-κB expression was reduced in LL and HL rats, accompanied by decreased MDA levels. It is possible that liraglutide inhibited NF-κB by upregulating PON3 expression, thereby protecting against oxidative stress. The lower MDA levels in HL rats compared to LL rats may be associated with the greater increase of PON3 expression in HL rats.
Obesity is involved in insulin resistance. Low-grade inflammation caused by obesity can impair the insulin receptor signaling pathway and is involved in the development of insulin resistance. Shih et al. found that PON3 knockout mice gained more BW and had a larger average gonadal adipocyte size than wild-type mice when fed the same high-fat diet. Moreover, PON3 knockout mice had significantly more subcutaneous, retroperitoneal and gonadal fat than wild-type mice. The authors suggested that PON3 can reduce obesity in mice by protecting the mitochondrial function of hepatocytes and white fat cells. These results indicate that low PON3 levels may be involved in the development of obesity. Decreased liver PON3 expression might also be involved in the development of obesity. Liraglutide enhanced PON3 expression, and BW was lower in LL and HL rats than in PC rats. The role of liraglutide in reducing BW might be related to its upregulation of PON3 expression.

In this study, PON3 expression was decreased in diabetic rats, and this was accompanied by abnormal lipid metabolism. Shih et al. found significantly increased TG levels in high-fat-fed PON3 knockout mice. On the other hand, PON3 deficiency can lead to changes in lipoprotein levels, including increased LDL-C and decreased HDL-C levels, in high-fat-fed mice. The reduced PON3 expression, increased TG and LDL-C levels, and decreased HDL-C levels in diabetic rats further suggest that reduced PON3 expression is associated with abnormal lipid metabolism. Liraglutide can improve lipid metabolism. In this study, PON3 expression was increased and abnormal lipid metabolism was improved after liraglutide treatment. It is possible that the effect of improved lipid metabolism was partially achieved by liraglutide increasing PON3 expression.

**Limitations**

Some limitations in our study should be considered. First, we have failed to investigate whether the PI3K/Akt pathway increases insulin sensitivity through PON3, although previous study showed that PI3K/Akt pathway is involved in the metabolic regulation of insulin through various mechanisms. Second, we have also failed to investigate the role of PON3 in obesity. Further in-depth study of the role of PON3 in PC could significantly contribute to understanding the development of abnormal glycolipid metabolism and obesity.

**Conclusions**

Liraglutide can increase PI3K, Akt and PON3 expression, and decrease NF-κB expression. The effect of liraglutide on improving insulin resistance and abnormal glycolipid metabolism in T2DM rats may be associated with increased liver PON3 expression.

**References**

1. Rull A, García R, Fernández-Sender L, et al. Serum paraoxonase-3 concentration is associated with insulin sensitivity in peripheral artery disease and with inflammation in coronary artery disease. Atherosclerosis. 2012;222(2):545–541. doi:10.1016/j.atherosclerosis.2011.11.021
2. Shih DM, Xia YR, Yu JM, Lusis AJ. Temporal and tissue-specific patterns of PON3 expression in mouse: In situ hybridization analysis. Adv Exp Med Biol. 2010;660:73–717. doi:10.1007/978-1-60761-350-3_8
3. Peng W, Zhang C, Lv H, et al. Comparative evaluation of the protective potentials of human paraoxonase 1 and 3 against CCl4-induced liver injury. Toxicol Lett. 2010;193(2):159–166. doi:10.1016/j.toxlet.2010.01.003
4. Zhu L, Shen Y, Sun W, et al. Paraoxonase 3 promotes cell proliferation and metastasis by PI3K/Akt in oral squamous cell carcinoma. Biomed Pharmacother. 2017;85(5):712–717. doi:10.1016/j.biopha.2016.11.084
5. Inoue K, Maeda N, Fujishima Y, et al. Long-term impact of liraglutide, a glucagon-like peptide-1 (GLP-1) analogue, on body weight and glycemic control in Japanese type 2 diabetes: An observational study. Diabetol Metab Syndr. 2014;6(1):95. doi:10.1186/1758-5996-6-95
6. Shao S, Nie M, Chen C, et al. Protective action of liraglutide in beta cells under lipotoxic stress via PI3K/Akt/FoxO1 pathway. J Cell Biochem. 2014;115(6):1166–1175. doi:10.1002/jcb.24763
7. Gao H, Zeng Z, Zhang H, et al. The glucagon-like peptide-1 analogue liraglutide inhibits oxidative stress and inflammatory response in the liver of rats with diet-induced non-alcoholic fatty liver disease. Biol Pharm Bull. 2015;38(5):694–702. doi:10.1248/bpb.b14-00505
8. Guo N, Sun J, Chen H, Zhang H, Zhang Z, Cai D. Liraglutide prevents diabetes progression in prediabetic OLETF rats. Endocrine J. 2013;60(1):15–28. doi:10.1507/endocrj.ej12-0094
9. Beek LV, Lips MA, Visser A, et al. Increased systemic and adipose tissue inflammation differentiates obese women with T2DM from obese women with normal glucose tolerance. Metabolism. 2014;63(4):492–501. doi:10.1016/j.metabol.2013.12.002
10. Rabingovitch A. An update on cytokines in the pathogenesis of insulin independent diabetes mellitus. Diabetes Metab Rev. 1998;14(2):129–151. doi:10.1002/scti.1099-0893(199806)14:2<129::aid-dmr208>3.0.co;2-v
11. Choi SE, Choi KM, Yoon IH, et al. IL-6 protects pancreatic islet beta cells from pro-inflammatory cytokines-induced cell death and functional impairment in vitro and in vivo. Transpl Immunol. 2004;13(1):43–53. doi:10.1016/j.trim.2004.04.001
12. Schweikert EM, Amort J, Wilgenbus P, et al. Paraoxonases-2 and -3 are important defense enzymes against Pseudomonas aeruginosa virulence factors due to their anti-oxidative and anti-inflammatory properties. Lipids. 2012;38(9):571–580. doi:10.1007/s11745-012-3836-7
13. Legarth C, Bastard JP, Auclair M, Maechi M, Capeau J, Caron M. Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte. Prevention by resiglitazone. Biochem Biophys Res Commun. 2003;311(2):372–379. doi:10.1016/j.bbrc.2003.10.013
14. Manea A, Manea SA, Gafencu AV, Raicu M. Regulation of NADPH oxidase subunit p22(phox) by NF-κB in human aortic smooth muscle cells. Arch Physiol Biochem. 2007;113(4–5):163–172.
15. Anrather J, Rachgummer I, Gadeo A, C. NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. Biochem. Biophys. Res Commun. 2006;281(1):567–566. doi:10.1016/j.bbrc.2005.12.002
16. Wang R, Peng L, Zhao J, et al. Gardnerimide A protects RGC-5 cells from H(2)(O)(2)-induced oxidative stress insults by activating PI3K/ Akt/eNOS signaling pathway. Int J Mol Sci. 2015;16(9):22350–22367. doi:10.3390/ijms160922350
17. Bautista E, Vergara P, Segovia J. Iron-induced oxidative stress activates Akt and ERK1/2 and decreases Dyrk1B and PRMT1 in neuroblastsoma SH-SY5Y cells. Trace Elem Med Biol. 2016;34:62–69. doi:10.1016/j.tem.2015.11.005
18. Bharathidevi SR, Babu KA, Jain N, et al. Ocular distribution of antioxidant enzyme paraoxonase & its alteration in cataractous lens & diabetic retina. *Indian J Med Res.* 2017;145(4):513–520. doi:10.4103/ijmr.IJMR_1284_14

19. Ferreira CER, Haas CS, Goularte KL, et al. Expression of paraoxonase types 1, 2 and 3 in reproductive tissues and activity of paraoxonase type 1 in the serum and seminal plasma of bulls. *Andrologia.* 2017;50(3):e12923. doi:10.1111/and.12923

20. Li PC, Liu LF, Jou MJ, Wang HK. The GLP-1 receptor agonists exendin-4 and liraglutide alleviate oxidative stress and cognitive and micturition deficits induced by middle cerebral artery occlusion in diabetic mice. *BMC Neurosci.* 2016;13(17):37. doi:10.1186/s12868-016-0272-9

21. Nandipati KC, Subramanian S, Agrawal DK. Protein kinases: Mechanisms and downstream targets in inflammation-mediated obesity and insulin resistance. *Mol Cell Biochem.* 2017;426(1–2):27–45. doi:10.1007/s11010-016-2878-8

22. Shih DM, Yu JM, Vergnes L, et al. PON3 knockout mice are susceptible to obesity, gallstone formation, and atherosclerosis. *FASEB J.* 2015;29(4):1185–1197. doi:10.1096/fj.14-260570

23. Kadowaki T, Ueki K, Yamauchi T, Kubota N. Snapshot: Insulin signaling pathways. *Cell.* 2012;148(3):624,624e1. doi:10.1016/j.cell.2012.01.034

24. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov.* 2005;4(12):988–1004. doi:10.1038/nrd1902