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METFORMIN EXERTS ITS ANTICANCER EFFECTS IN PART, PROBABLY THROUGH ELEVATION OF KLOTHO

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ABSTRACT

Metformin (1, 1-Dimethy- Biguanide) is used extensively in treatment of hypeglycemia and diabetes, PCOS, weight control, and modulation of physiological activities. This drug is lipophilic and liver is its prefered target organ inside which mitochondria are affected by metformin initially. Metformin has anticancer properties and exerts its anticancer effect in a number of ways. In one pathway, it inhibits cancer cell growth through Lowering glucose, insulin and IGFs levels. In another pathway, its anticancer effects are exerted by activation of AMPK. Another probable way for anticancer action of metformin is regulation of fatty acid synthesis through AMPK. Metformin could also act as an anticancer through inhibition of pro-inflammatory factors and angiogenesis in tumoral tissues. Some other anticancer effects of metformin are put into effect through down- regulation of expression of the on coprotein HER2 (erb B-2), induction of cessation of cell cycle, and activation of apoptosis pathways. Our novel finding indicates that metformin could act as anticancer agent through increasing expression of klotho gene. Given the effects of metformin on insulin, gucose, and IGF1 levels and also disruption of functions of insulin and IGF1 by klotho, we hypothesized a possible relationship between metformin and Klotho.

In this case- control study 45 women with PCOS who referred to Infertility center of Jahad – e- Daneshgahi in Ardabil, Iran from March 2013 through March 2014, were selected in a accordance with NIH criteria. 45 healthy women were also selected as controls. BMIs were calculated as well as insulin resistance index based on HOMA- IR model. Klotho serum levels were measured with an ELISA kit made by Glory. In patients group the measurements were repeated after one month of therapy with metformin.

Patients showed various improvements after one month of treatment with metformin. Patient's weights decreased ($p<0.05$). Fasting glucose levels and insulin resistance decreased significantly ($p<0.05$). Hormonal assays showed significant falls in serum levels of insulin and ris e in klotho levels. BMIs did change significantly.

Our study showed that serum levels of secretory klotho in PCOS women are not significantly different from healthy women's klotho levels. Furthermore we showed that metformin increases secretory form of klotho in PCOS women, significantly. In our study metformin increased klotho without affecting BMIs. Given the antitumoral properties of klotho, the present study, probably for the first time, declares that metformin probably exerts its anticancer effects- at least in breast cancer- through increasing the secretory form of klotho.

Keywrds: Anticancer- Tumor -Metformin- Klotho- PCOS

INTRODUCTION

Metformin (1,1dimethylbiguanide) a biguanide derivate, is the most widely prescribed drug to treat hyperglycaemia in individuals with T2D and is ecommended, in conjunction with lifestyle modification (i.e. diet, weightcontrol and physical activityas a first-line oral therapy in the recent guidelines of the ADA (American Diabetes Association) and EASD (European Association of the Study of Diabetes) **[1, 2]**. Shu and collegues in 2007 showed various isoforms of OCT (orgnic-cation transporter) possibly facilitate transportation of metformin through cell membrane. (OCT_1 in liver OCT_2 in kidneys) The preferential action of metformin in hepatocytes is due to the predominant expression of OCT1 (organic cation transporter 1), which has been shown to facilitate cellular uptake of metformin. Consistent with this, accumulation of metformin in the liver has been shown to be higher than in other tissues, reaching high micromolar concentrations in the periportal area. Furthermore, deletion of the *Oct1* [*Slc22a1* (solute carrier family 22member 1)] gene in mice dramatically reduces metformin uptake in hepatocytes, and human subjects carrying polymorphisms of the *SLC22A1* gene display an impaired effect of metformin in lowering blood glucose level. Upon entering the cell, metformin affects mitochondria initialy.It enters the mitochondria by its hydrocarbon chain and disrupts complex-1 of electron transport chain **[3, 4]**. More recently, metformin's antitumoral effects have attracted researcher’s interests. Recent prospective and case-control studies conducted on large cohorts have confirmed that T2D is associated with a significantly increased risk of cancer mainly affecting the breast, colon, prostate, kidney and pancreas **[5]**. This increased risk has been attributed to the growth-promoting effect of chronic

elevated plasma insulin levels **[6]**. Insulin resistance and the resultant hyperinsulinaemia might indeed promote carcinogenesis directly through the insulin receptor or indirectly by increasing the levels of IGFs, steroid sex hormones, inflammatory processes and disrupting adipokines homoeostasis **[6]**. However, additional explanations for this association may be proposed, such as the role of persistent elevated plasma glucose levels. **[7]**. Metformin lowers cancer risk in diabetes type-2 patients through lowering insulin and glucose plasma levels **[15]**. Given the epidemiological evidence between T2D and an increased risk of cancer, the impact of metformin therapy on cancer risk and cancer-related mortality has been evaluated in the first pilot case-control study with a cohort of 12 000 T2D patients **[8]**. Metformin therapy was associated with a reduced risk of cancer (odds ratio of any exposure to metformin was 0.79). Furthermore, the authors found a dose–response relationship between the duration of exposure to metformin and cancer incidence **[8]**. In Greek mythology, three goddesses determine life span of every mortal by controlling the thread of life. They are Klotho, Lachesis, and Atropos who spins, measures, and cuts the thread of life, respectively. The klotho gene, named after the spinner, was identified in 1997 as a gene mutated in the klotho mouse, which displays extremely shortened life span with multiple disorders resembling human premature-aging syndromes **[9]**. The klotho gene encodes a single-pass transmembrane protein. The extracellular domain of Klotho protein is clipped just above the plasma membrane by membrane-anchored proteases ADAM10 and ADAM17 to generate a secreted form of Klotho protein. In fact, the entire Klotho ectodomain is detectable as a single peptide in the blood, urine, and cerebrospinal fluid **[10, 11]**. Thus, Klotho protein exists in two forms. One is the transmembrane form expressed primarily in renal tubular cells, and the other is the secreted form circulating in the blood **(Figure 1)**.

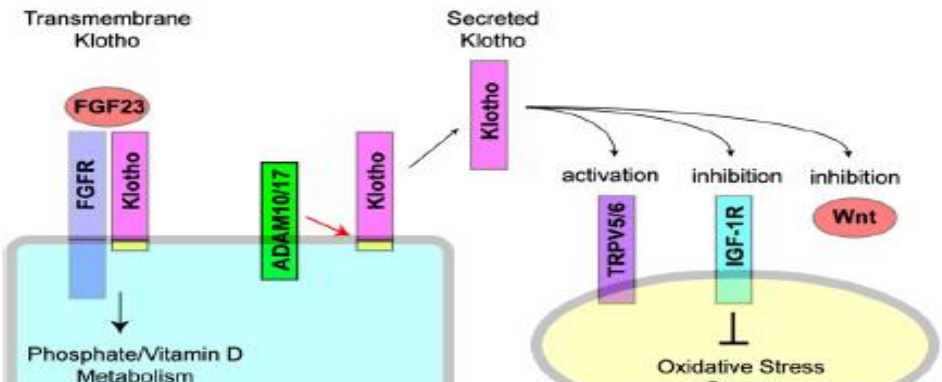

Figure 1: Function of Klotho protein. The transmembrane Klotho forms a complex with FGF receptor (FGFR) and functions as a co-receptor for FGF23 and plays a crucial role in the regulation of phosphate and vitamin D metabolism in the kidney. On the other hand, the transmembrane Klotho is clipped by membrane-anchored proteases ADAM10 and ADAM17 just above the plasma membrane. The entire extracellular domain of Klotho is then released into blood, urine, and cerebrospinal fluid. The secreted Klotho protein has a putative sialidase activity that modifies glycans of calcium channel TRPV5 on the cell surface. A imilar mechanism may explain the inhibitory effect of secreted Klotho on growth factors including insulin, IGF-1, and Wnt. The ability of secreted Klotho to inhibit IGF-1 signaling maycontribute to the anti-oxidative stress and anti-cancer properties of Klotho.

Ample clinical and laboratory data indicate a critical role for insulin/IGF-1 signaling in breast cancer. Notably: 1) increased serum insulin levels are associated with adverse prognosis in

breast cancer **[12]**; 2) high circulating IGF-1 levels are associated with increased risk of pre-menopausal breast cancer **[13]**; and 3) inhibition of insulin/IGF-1 signaling inhibits growth of breast cancer cells **[14]**. Since secreted Klotho protein inhibits activation of insulin/IGF-1 receptors, Klotho may function as a suppressor of breast cancer. Consistent with this notion, immunohistochemical analysis of human breast tissue arrays showed that normal breast epithelial cells express. Klotho protein, whereas its expression is significantly decreased or lost in ductal carcinoma in situ and invasive ductal carcinoma. Aforementioned studies led us to hypothesizing a relationship between klotho and metformin.

MATERIALS AND METHODS

This is a case- control study in which 45 women with PCOS referring to Infertility center of Jadad Daneshgahi in Ardabil, Iran were selected in accordance with NIH criteria. 45 other women who were analogous to patients age- wise and BMI – wise and did not have clinical and laboratory features of PCOS, were also selected as controls from population who referred to gynecology clinic for annual Pap smear test and clinical evaluations. Remainder of serum was frozen immediately in 80 degrees Celsius for measurement of klotho. All patients and controls underwent clinical examination and their medical histories were taken. BMIs were calculated by division of weight in kgs by the square of height in squar meters (m^2). 5 mililiters of venous blood Was drawn from each specimen following 9 10-12 hour fasting period between 9 and 9:30.a.m.Routin measurements were

performed on the same day and the remainder of serum was frozen immediately in -80 degrees Celsius for measurement of klotho. Hormonal assays were done by ELISA method and a kit made by Glory used for measurement of klotho in a private medical laboratory in Ardabil, Iran.Data analysis was done using SPSS software edition 20.All results were categorized in standard deviation averages. Paired sample t-test was used to compare means of groups and pearson correlation coefficient was used determine relationships among quantitative variables in groups. A p- value less than 0.05 were considered meaningful.

Table1: Comparison of Demographic and biochemical features of patients and controls

Measured parametrs	controls n=45	PCOS women before metformin therapy n=45	PCOS women after metformin therapy n=45
Age	36/2±3/13	30/91±2/26	30/91±2/26
Hight(cm)	161/75±4/15	161/36±6/14	161/36±6/14
weight(kg)	68/1±8/28	71/95±8/92	70/45±8/66
BMI(kg/m^2)	26/09±3/48	28/36±3/85	27/30±3/55
Insulin(mlU/ml)	7/96±2/17	11/52±2/24	7/03±2/06
Insulin Resistance (IR)	1/90±0/54	2/67±0/49	1/50±0/48
FBS (mg/dl)	94/45±5/22	93/54±4/63	86/18±4/85
Klotho(ng/L)	3/64±1/20	4/01±0/97	5/43±1/21
leptine(ng/L)	22/77±10/60	34/74±7/88	28/40±6/92

RESULTS

Patients showed various improvements after one month of treatment with metformin. Patient's weights decreased ($p<0.05$). Fasting glucose levels and insulin resistance decreased significantly ($p<0.05$). Hormonal assays showed significant falls in serum levels of insulin and risa in klotho levels. BMIs did change significantly. Measurement of klotho levels in 45 patients showed that its level increased from 4.01(ng/L) to 5-43. (ng/L) **Table 1**.

This study indicated that treatment with metformin causes significant drops in glucose and insulin levels, weight, and insulin resistance and rise in klotho levels.

Although the underlying mechanisms are not yet completely elucidated, the association between metformin and a reduced risk of cancer in T2D patients may simply be explained through the action of metformin on the improvement in blood glucose and insulin levels **[15]**. Accordingly, prevention of tumour growth in animal models with diet-induced hyperinsulinaemia is attributable to reductions in circulating insulin levels **[16, 17]**. Given that hyperinsulinaemia is associated with increased levels of IGF-1, it is possible that the metformin-lowering effects on serum insulin and IGF- 1 levels might explain, at least in part, its therapeutic efficacy.

This hypothesis is particularly relevant in light of recent studies showing that calorie restriction, which lowers insulin and IGF-1

levels, induces a dramatic decrease in the incidence of cancer in rodent models **[18]**.

However, a decrease in insulinaemia is not always correlated with metformin efficacy as shown in PTEN (phosphatase and tensin homologue deleted on chromosome 10) +/− , HER-2/neu and APC (adenomatous polyposis coli) min/+ mouse tumour models, indicating an insulin-independent antitumoral action of metformin . Hence metformin appears to have a direct action on tumour growth both *in vitro* and *in vivo* by a mechanism involving activation of the LKB1/AMPK pathway and subsequent modulation of downstream pathways controlling cellular proliferation **(Figure 1)**. AMPK knockdown by siRNA (small interfering RNA) or AMPK inhibitors partially revert the antiproliferative action of metformin in breast and ovarian cancer cells **[19–20]**. Furthermore, the antitumoral action of metformin was significantly reduced in mice displaying a decrease in LKB1 expression **[21]**. Another mode of action of metformin might be through an AMPK-mediated regulation of fatty acid synthesis. Indeed, cells derived from prostate, breast and colon cancers constitutively overexpress FAS, a key enzyme for *de novo* fatty acid biosynthesis, which has been associated with a malignant phenotype. Interestingly, it has been observed that a reduction in FAS and ACC expression by AMPK activation diminishes the viability and growth of prostate cancer cells **[22]**. Another potential mechanism is based on the positive impact of metformin on chronic inflammation **[23]**, a major contributory factor to cancer development and progression. Emerging results showing the capacity of AMPK to inhibit the inflammatory responses **[24]** suggest that metformin may also target the inflammatory component present in the microenvironment of most neoplastic tissues, leading to tumour reduction. In addition, inhibition of neoplastic angiogenesis by metformin might also participate in the reduction of tumour growth **[25]**.

Although these results suggest a pivotal role of LKB1/ AMPK signalling, the antineoplastic action of metformin could also be independent of AMPK activation. Indeed, metformin was reported to decrease the expression of the oncoprotein HER2 (*erbB-2*) in human breast cancer cells via a direct and AMPK-independent inhibition of p70S6K1 (p70 S6 kinase 1) activity **[26]**. Metformin also exerts its anticancer effect through induction of cell- ycle arrest in prostate cancer cell lines via a decrease in cyclin D1 protein expression and an increase in REDD1 (regulated in development and DNA damage response 1) expression in a p53-dependent manner **[27]**.

In addition to the inhibition of cancer cell proliferation, metformin has been shown to promote cell death of some cancer cells

through the activation of apoptotic pathways by both caspase-dependent and caspase-independent mechanisms **[28, 29]**.

In addition, several lines of experimental evidence support the notion that Klotho functions as a tumor suppressor. 1) Forced expression of Klotho in breast cancer cell lines reduces their proliferation. 2) Knockdown of endogenous Klotho expression in MCF-7 breast cancer cells promotes their proliferation. 3) Forced expression of Klotho in MCF-7 cells attenuates GF-1-induced phosphorylation of the IGF-1 receptor, IRS-1, AKT1, and ERK. Similar effects were observed when MCF-7 cells are treated with recombinant secreted Klotho protein. 4) knock-down of endogenous Klotho in MCF-7 cells enhanced IGF-1-induced AKT hosphorylation. 5) Inhibition of IGF-1 signaling by Klotho increased expression of CAAT/enhancer-binding proteins (C/EBP). These transcription factors are known to be down-regulated by IGF-1 and identified as breast cancer growth suppressors. All these observations are consistent with the hypothesis that Klotho may be a suppressor of breast cancer. It remains to be determined whether the anti-breast cancer activity of Klotho depends primarily on its ability to suppress IGF-1 signaling or on other unknown mechanisms **[30]**.

Marie and colleagues in 2013 demonstrated the novel finding that the levels of the anti-aging hormone klotho are reduced in the plasma of both obese and r-AN patients . this may strengthen the notion that maintaining a normal BW is important in preventing age –related disease and in achieving longevity **[31]**.

In this study we observed no meaningful difference between levels of secretory klotho in PCOS and healthy women. Also, metformin therapy increases secretory klotho in PCOS women, significantly.

CONCLUSION

Metformin could increase klotho levels in obese individuals through decreasing their BMIs. Our study showed that metformin could also act similarly on klotho levels without affecting patient's BMIs. Given the antitumoral properties of klotho, the present study, probably for the first time, declares that metformin probably exerts its anticancer effects- at least in breast cancer- through increasing the secretory form of klotho.

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