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Original article

Metformin Effects on Pancreatic Cancer Cells

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ABSTRACT

Background and aims. *Pancreatic cancer is one of the most common causes of cancer related deaths around the world. A number of transcription factors and cytokines are associated with pancreatic cancer formation. These proteins play a momentous role in the inflammatory response that may end in cancer formation. Metformin, an oral antidiabetic agent from the biguanide class has been reported to possess significant antitumor and anti-inflammatory activities. The present study was conducted to investigate the effects of this medication on pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinoma cells.* ***Methods****. We worked with pancreatic intraepithelial neoplasias (PanIN) and pancreatic ductal adenocarcinoma (PDAC) cell lines, which have a characteristic K-Ras, and p53 mutation. In order to determine the effect of metformin on expression of (TNFα, IL6, IL1β and IKKβ) genes in the two cell lines (PDAC and PanIN), 5 X105/ml cell suspensions of these cell lines were seeded into 3-wells plate. The first well was the control well i.e., untreated well and the remaining two wells were treated with 10μM of the drug (Metformin).* ***Results****. The proliferation assay results showed that the antidiabetic drug inhibits cancer cells growth in a concentration and time-dependent manner, in both pancreatic cancer cell lines. In addition, treatment with Metformin resulted in downregulation of TNFα and IKKβ genes in the PanIN cell line. However, Metformin up-regulated TNFα gene and down-regulated mRNA IKKβ in PDAC cells.* ***Conclusion****. Taken together, these findings show that Metformin possesses inhibitory and anti-inflammatory effects on pancreatic cancer cells and it may represent a novel approach to prevent or treat this kind of cancer.*

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INTRODUCTION

A significant body of evidence supports the conclusion that inflammation is a vital component of tumor development. Many malignancies arise from sites of chronic irritation and inflammation. It is now becoming clear that the tumor microenvironment, which is largely composed of inflammatory cells, is an essential participant in the tumorigenic process such as the increase in proliferation, migration and survival [1]. For example, many studies have shown that there is a strong association between chronic inflammatory bowel diseases and the risk of developing colon cancer [2]. In addition, sporadic and hereditary types of chronic pancreatitis are connected with an increased risk of developing pancreatic adenocarcinoma [3]. The combined increase in cellular proliferation and genomic damage, both of which are seen with inflammation, are both involved in strongly stimulating the malignant transformation of pancreatic cells. Mediators of the inflammatory pathway such as COX-2, NF-kB, cytokines and reactive oxygen species have been observed to increase cell proliferation, stimulate oncogene expression and cause loss of tumor suppressor function; all of which may lead finally to pancreatic malignancy [4]. Diabetic patients have a high incidence of developing pancreatic cancer [5]. Along this line, drug such as Metformin are best characterized by their insulin-sensitizing action and have been used in the treatment of diabetes where they also have anti-inflammatory activity [6]. Patients taking metformin also show a lower risk of pancreatic and prostate cancer incidence [7]. This study will assess the impact of this drug on pancreatic cancer cells line to address the changes in inflammatory processes on these cells.

Pancreatic cancer is one of the most common causes of cancer related deaths. The symptoms of pancreatic cancer are generally nonspecific and might occur at a late stage of the disease [8]. As a result, pancreatic cancer is usually diagnosed at an advanced stage, usually after the tumor has already metastasized and spread to other parts of the body[9].Pancreatic

cancer is considered an insensitive disease to pharmacological and radiological therapy and also comes back after curative surgery [10]. All these factors contribute to the poor prognosis associated with this cancer. Chronic pancreatic inflammation may present at an early phase of the malignancy. Present epidemiological studies suggest patients with both hereditary and sporadic chronic pancreatitis have an increased risk of pancreatic cancer [11]. Persistent inflammation in pancreatic tissue could lead to malignant transformation of ductal cells causing dysplasia and ultimately leads to cancer formation (Fig.1.). Similar to other cancers, in pancreatic cancer the mutation of proto-oncogenes and the loss of tumor suppressor genes both have been implicated in pancreatic ductal carcinoma development. K-ras mutations are often found in most of cases of pancreatic adenocarcinomas; 90% of cases of the cancer have this kind of mutation with the additional loss of the tumor suppressor protein p53 [12]. In addition, P16 and DPC4/SMAD mutations are considered to be important etiological factors involved with pancreatic cancer formation [13].

Metformin is an oral antidiabetic agent from the biguanide class. It is the first choice of drug for treatment of type 2-diabetes. It works by increasing liver and tissue sensitivity to insulin action as well as reducing hepatic glucose production. Many studies have suggested that metformin can decrease the inflammatory reaction in some cases of polycystic ovary syndrome by reducing the inflammatory markers in the plasma, including soluble intercellular adhesion molecules, vascular cell adhesion molecules-1, macrophages migration inhibitory factor, and C-reactive protein [14]. Recent data has found that metformin can exercise direct anti-inflammatory action by inhibiting NF-kB during blockade of the PI3K–Akt pathway in vascular walls [15]. Metformin activates the AMP-activated protein kinase, which acts to induce glucose uptake in muscles. Targeting AMP-activated protein kinase needs LKB1, which is well known as a tumor suppressor. The relationship between LKB1and metformin might then be an explanation for the possible beneficial effects of metformin on cancer development [16].

Previous clinical studies found that cancer incidence was low in patients exposed to metformin compared with unexposed patients [17]. Metformin has also been found to be beneficial in patients with specific kinds of cancer. For instance, type 2 diabetic patients who are taking neoadjuvant chemotherapy and metformin for treatment of breast cancer were more likely to have a full remission compared with patients not receiving metformin treatment [18]. The aim of this study is to investigate the potential role of metformin on pancreatic cancer cell lines on gene expression of inflammatory mediator and cytokines.

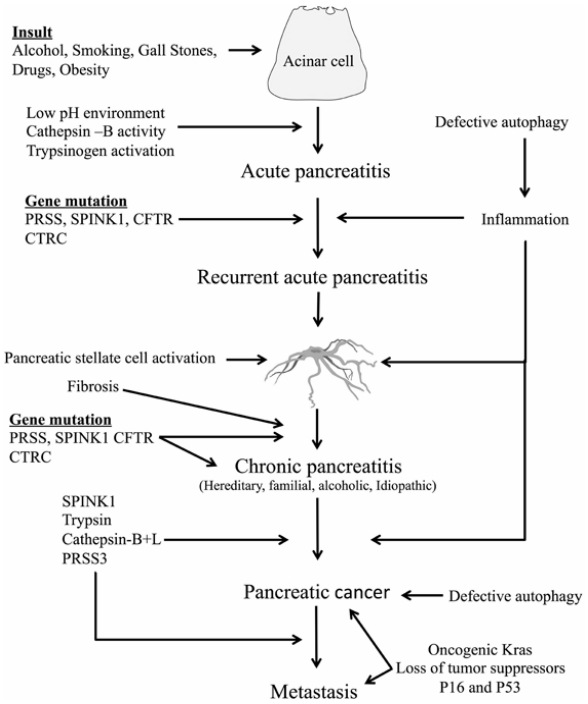


Figure 1: The mechanism of inflammation formation and the development of pancreatic cancer. The image adopted from https://www.frontiersin.org/articles/10.3389/fphys.2013.00415/full

METHODS

Cell lines

We worked with pancreatic intraepithelial neoplasias (PanIN) and pancreatic ductal adenocarcinoma (PDAC) cell lines, which have a characteristic K-Ras, and p53 mutation. These cells are taken from the embryonic pancreas progenitor of Pdx1Cre LSL-KrasG12D mice model (The cell lines are obtained from Dr David Tuveson, Cambridge University).

Preparation of Cell Culture Medium

Cell lines were routinely grown in Dulbecco's modified Eagle (DMEM) media (500ml) supplemented with 4.5 g/liter glucose and 4 mM L-glutamine purchased form (PAA Company) supplemented. The growth medium was prepared by adding 50 ml heat inactivated (56oc for 1 hour) fetal calf serum (FCS) and 5 ml of 100X penicillin/streptomycin mixed solution to Dulbecco's modified Eagle's medium. The final concentration of ingredients is 10% FCS, 2.0Mm L-glutamine, 1% penicillin/ streptomycin.

Cell Culture Conditions

The cell lines were sub cultured in tissue culture flask (T175 flask) and were grown under standard conditions 370C, 5% CO2. The cells were passed every 3-4 days with the prepared cell culture medium.

Cell proliferation assay

To assess the effect of the drug on the proliferation/survival of PanIN and PDAC cell lines, the cell were plated at a density of 2x105 per well in 3-well plates and the cells were treated with 4 different concentration of metformin (5,10,15,20µM) the treated cells then incubated in 5% CO2 atmosphere at 37C0 for two time points (24hr & 48hr). The old media was removed and replaced with about 500µl of trypsin, after 5 min of incubation, the action of trypsin was stopped by adding 500 µl of fresh media containing 10% FCS and 1% of P/S. Then the number of cells was counted by using Vi-cell XR automated cell viability analyzer (Beckman Coulter). All experiments were repeated three times. Means and standard deviations were presented in the figures. (Refer to appendix).

RNA Purification and RT-PCR

In order to determine the effect of metformin on expression of (TNFα, IL6, IL1β and IKKβ) genes in the two cell lines (PDAC and PanIN), 5 X105/ml cell suspensions of these cell lines were seeded into 3-wells plate. The first well was the control well i.e., untreated well and the remaining two wells were treated with 10µM of the drug (Metformin). The treatment was carried out by incubation at 37C0 for 6 hours.

RNA Extraction

RNA is transcribed from DNA via enzymes called RNA polymerases along with other enzymes like DNA Helicase and Protein Kinase. RNA is necessary for protein synthesis. Frequently, RNA that has been extracted from the tissue or cells is further processed hence can be used to study the expression levels of specific genes of interest. The process of purification of the RNA needs careful handling because RNA is very susceptible to degradation by the RNase enzyme. Moreover, during one of the isolation stages, the RNA could become contaminated with DNA.

Total RNA was extracted using the Qiagen RNeasy Mini kit. Three extractions were performed for each cell line, and total RNA was pooled at the end of the RNeasy protocol. All steps were performed according to the manufacturer's instructions. Briefly, the cells were disrupted by using RLT buffer (guanidine thiocyanate) the lysate was then pipetted into QIA shredder spin column to be centrifuged for specific time (about 5 min). Next 1 volume of 70% of ethanol was added into lysate. The homogenate lysate was then transferred into the RNeasy spin column. After 15s of centrifugation, the column was then treated with DNase digestion to eliminate genomic DNA contamination. (Refer to appendix).

RPE buffer (guanidine hydrochloride) was added to the column followed by centrifuging (This step was repeated two times). The final step in RNA extraction was by adding RNase-free water directly to the spin column membrane followed by final centrifugation.

cDNA synthesis and RT-PCR

The polymerase chain reaction (PCR) is often used to amplify a piece of DNA that has been synthesized from an RNA template. This method gets its name from the enzyme DNA polymerase (reverse transcriptase) that is used in the reaction

to synthesize a single strand of DNA, identified as complementary DNA (cDNA). The synthesized cDNA is used as a template in the polymerase chain reaction (PCR). Throughout the reaction, specific regions of cDNA are amplified with the help of primers. The primers are short, specific sequences of oligonucleotides that unite to complementary sequences in cDNA. In the PCR reaction, one time the primers have bound the DNA polymerase extends them along the length of the cDNA by adding nucleotides complementary to those in the cDNA. These newly synthesized cDNA templates are then used themselves as new templates in the polymerase chain reaction that increases the amount of cDNA template.
Two micrograms of the total RNA sample were subjected to cDNA synthesis. Briefly, in a sterile PCR tube the following aliquots were added: 2µl of random hexamers, 10µl DEPC-treated water and 2µl of RNA. The mixture was then heated at 75ºC for 5 min and chilled in ice for 5 min. In the next step, about 11.6µl of mixture of (5µl of M-MLV reverse transcriptase buffer, 5µl of nucleotide pool, 1µl of M-MLV reverse transcriptase enzyme and 0.6µl of RNasine) were added to the PCR tube that containing the RNA and other mixtures. The tube was then incubated for 10 min at room temperature followed by 50 min in 40C0. Finally, 20µl of DEPC water was added to the sample in the tube. The Samples were subjected to real-time PCR analysis on an Applied Biosystems PRISM 7700 Sequence Detection System (Applied Biosystems).
The RT-PCR cycle was repeated 25-35 times. The primer sequences for the PCR reaction are shown below:
TNF- α ; Forward, 5'-AAA GCA TGA TCC GAG ATG TG-3'; Reverse, 5'-AGC AGG AAT GAG AAG AGG CT- 3', IL-6: Forward, 5'-CCG GAG AGG AGA CTT CAC AG-3'; Reverse, 5'-AGA ATT GCC ATT GCA CAA C-3', IL-1β; Forward, 5'-CAT CTT TGA AGA AGA GCC CG-3'; Reverse, 5'- GGG ATT TCG TTG TTG CTT GT-3'. All the assays were performed in triplicate and normalized with 18S internal control.

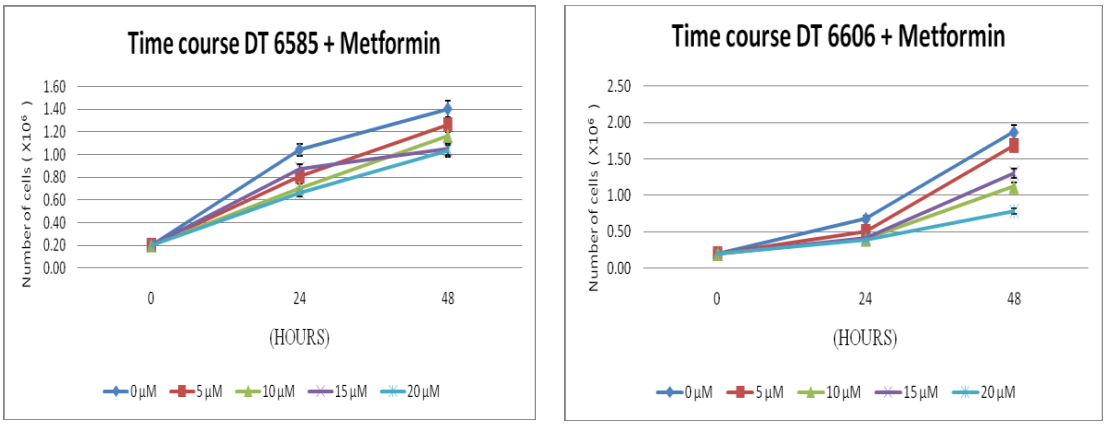
Statistical Analysis

Data represented in mean ± SD from 3 independent experiments value was taken as level of significance at <0.05. Statistical analysis was carried out in IBM SPSS version 22.

RESULTS

Metformin inhibits pancreatic cancer cell proliferation in a concentration and time- dependent manner.

To assess the effect of metformin on the proliferation/survival of the two cancer cell lines (PDAC and PanIN), the cells were treated with 4 different concentrations of the metformin (5, 10, 15,20µM) for two time points (24hr & 48hr). Our result revealed that there is significant inhibitory action made by metformin on the cells in both cell lines. In addition, the reduction of the cellular growth rate was also in a dose-dependent manner as by increasing concentration of drugs the proportion of cell growth was declined. Moreover, the magnitude of this inhibition increased with time, as the number of alive cells was lesser after 48 hr of incubation in comparison with the 24hr treated group as shown in figure (Figure 2).



A B

Figure 2: (a) Effect of Metformin on PanIN (DT6585) cell line. Cells were treated with 0-20 µM of Metformin for two time points (24hr & 48hr). Data represented are mean ± SD from 3 independent experiments. (b) Effect of Metformin on PDAC (DT6606) cell line. Cells were treated with 0-20 µM of Metformin for two time points (24hr & 48hr). Data represented are mean ± SD from 3 independent experiments.

The effect of Metformin on expression of TNFα gene in the PanIN cell line.

To explore the molecular mechanisms behind the inhibitory action induced by the drug we investigated their effects on mRNA expression of TNFα protein that are intensively related to tumor growth and migration pancreatic cancer by using RT-PCR technique. The data revealed that this drug downregulates TNFα gene in the pancreatic intraepithelial neoplasias cell line (PanIN). This concludes that Metformin has an effect on mRNA expression of this cytokine (Figure 3).

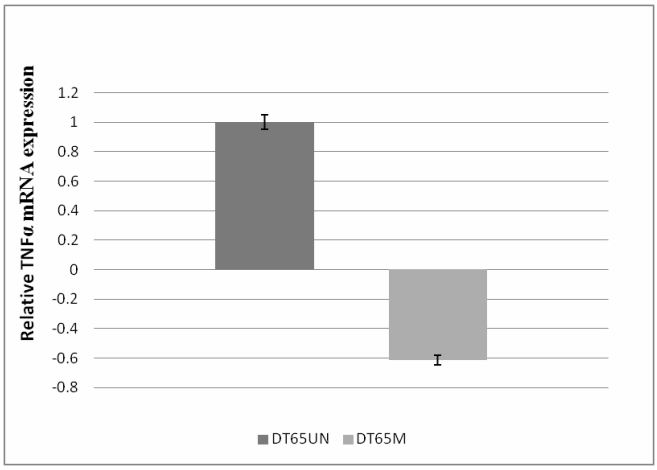


Figure 3: Effect of Metformin on TNFα gene expression in PanIN (DT6585) cell line. Cells were treated with 10 µM of metformin for 6 hours determined by RT-PC Data represented are mean ± SD from 3 independent experiments. (UN-untreated cells).

Antidiabetic drug Metformin has an effect on expression of TNFα gene in PDAC cell line

The study so far has shown that gene expression of inflammatory cytokine TNFα in the PanIN cell line which is treated with and metformin was down- regulated (Figure 5). So, in the next step, we need to investigate the effects of these drugs on TNFα gene expression in the other cell line (PDAC) also by using RT-PCR technique.

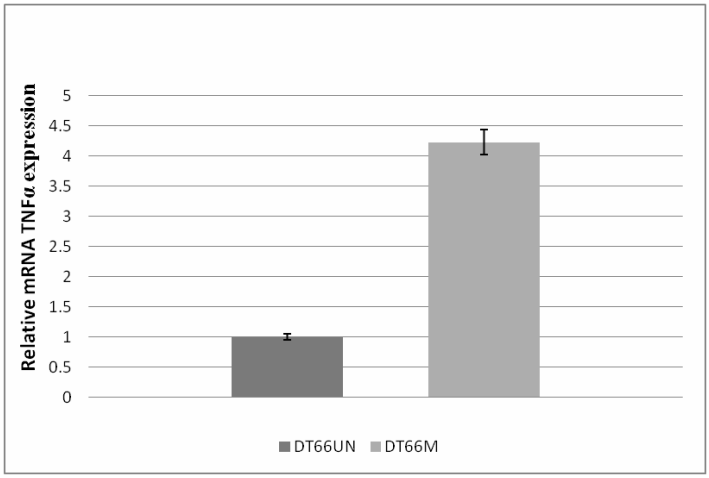


Figure4: Effect of Metformin on TNFα gene expression in PDAC (DT6606) cell line. Cells were treated with 10 µM of metformin for 6 hours. TNF α mRNA levels were determined by RT-PCR. Data represented are mean ± SD from 3 independent experiments. (UN-untreated cells).

The result demonstrates (Figure 4) that the cells which are treated with Metformin show significant up-regulation of mRNA TNFα cytokine compared with untreated cells. This result indicates that the drug demonstrates inflammatory effects on the pancreatic ductal adenocarcinoma cells.

DISCUSSION

Epidemiological studies have shown that metformin is associated with a low risk of pancreatic cancer in diabetic patients [19]. Moreover, previous studies have shown that the drug are also involved in anti-inflammatory action [20]. In the present study, these drugs were shown, for the first time, to exert a growth inhibitory effect on pancreatic cancer cell lines (PanIN and PDAC). The proliferation assay showed that the drug reduces the growth in both cells in a concentration- and time-dependent manner as shown in Figure 3 and Figure 4. These results strongly suggest that this drug has a cytostatic rather than cytotoxic effect on PanIN and PDAC cell lines as this drug works to stop cells from multiplying. However, its effect was clearer in the PanIN (DT6585) cell line. This is probably due to the different velocity of cellular growth in these cells (PanIN and PDAC). Our in vitro studies further verified the in vivo results which showed that these drugs have an effect on the nude mouse xenograft model [21]. To obtain a better understanding of the molecular mechanism by which these drugs function as anticancer agents, we tried to explore the anti-inflammatory action of these drugs by looking at the change of expression of some cytokines and transcriptional factors (TNFα, IL6, IL1β and IKKβ) genes after 6 hours of treating with these drugs. As expected, our results expressly showed that treatment with metformin significantly down-regulated the expression of TNFα gene in the PanIN (DT6585) cell line as shown in Figure 5, which was more downregulated in metformin-treated cells. TNFα is an important internal cytokine that has a central role in immune homeostasis and inflammation. Previous studies have already shown that metformin has a down- regulatory effect on mRNA TNFα in human monocytes [22]. However, this first study reveals their action in pancreatic cancer cells.

In the PDAC cell line, the result was slightly different, the cells which were treated with metformin represent down-regulation of the TNFα gene as shown in Figure 6. The most interesting finding of this study is the observation that the expression of IKKβ which is considered part of the IKK complex in the conventional pathway of NF-kappa-B (NF-kβ), is a transcription factor that regulates the expression of a variety of inflammatory, oncogenic and apoptotic genes. Activation inhibitors of NF-kappa-β by phosphorylation lead to the dissociation of the NF-kappa-B/ inhibitor complex and eventually the degradation of the inhibitor. Surprisingly, the mRNA of IKKβ was down-regulated by the effect of the drugs in the PanIN cell line. Metformin had a strong down-regulatory effect on IKKβ in pancreatic intraepithelial neoplasias (PanIN). The cells which were treated with metformin represent down-regulation of the IKKβ gene as shown in Figure 8. All these findings illustrate that metformin has an inhibitory and anti-inflammatory effect on the PanINs and PDAC cells.

CONCLUSION

In recent years, more subtypes of cancer are being discovered and understood at a genetic and molecular level. The results of this study present evidence for remarkable efficiency of rosiglitazone and metformin to inhibit pancreatic cells growth in vitro, and highlight their use in the treatment and prevention of pancreatic cancer. Despite the difference in mechanism of action of these drugs in the treatment of diabetes, the present study extends our knowledge about the role of these drugs in gene expression of cytokines and transcriptional factors which play an important role in preventing the development of pancreatic cancer.

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REFERENCES

1. Mantovani A, Allavena P, Sica A, and Balkwill F. Cancer-related inflammation. Nature, 2008;454:436–444.
2. Jain SK, Peppercorn MA. Inflammatory bowel disease and colon cancer: a review. Digestive Diseases and Sciences, 1997;15(4–5):243–52.
3. Lowenfels AB, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andrén-Sandberg A, Domellöf L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. N Engl J Med. 1993 May 20;328(20):1433-7.
4. Farrow B, Evers BM. Inflammation and the development of pancreatic cancer. Surg Oncol. 2002 May;10(4):153-69.
5. Huxley R, Ansary-Moghaddam A, Berrington de González A, Barzi F, Woodward M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. Br J Cancer. 2005 Jun 6;92(11):2076-83.
6. Currie CJ, Poole CD, Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. Diabetologia. 2009 Sep;52(9):1766-77. doi: 10.1007/s00125-009-1440-6. Epub 2009 Jul 2. PMID: 19572116.

7. Wright JL, Stanford JL. Metformin use and prostate cancer in Caucasian men: results from a population-based case-control study. Cancer Causes Control. 2009 Nov;20(9):1617-22. doi: 10.1007/s10552-009-9407-y. Epub 2009 Aug 4. PMID: 19653109; PMCID: PMC2767519.
8. Gullo L, Tomassetti P, Migliori M, Casadei R, Marrano D. Do early symptoms of pancreatic cancer exist that can allow an earlier diagnosis? Pancreas. 2001 Mar;22(2):210-3. doi: 10.1097/00006676-200103000-00017. PMID: 11249079.
9. Lillemoe KD, Yeo CJ, Cameron JL. Pancreatic cancer: state-of-the-art care. CA Cancer J Clin. 2000 Jul-Aug;50(4):241-68. doi: 10.3322/canjclin.50.4.241.
10. Warshaw AL, Gu ZY, Wittenberg J, Waltman AC. Preoperative staging and assessment of resectability of pancreatic cancer. Arch Surg. 1990 Feb;125(2):230-3.
11. Lowenfels AB, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andrén-Sandberg A, Domellöf L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. N Engl J Med. 1993 May 20;328(20):1433-7.
12. Hruban RH, van Mansfeld AD, Offerhaus GJ, van Weering DH, Allison DC, Goodman SN, Kensler TW, Bose KK, Cameron JL, Bos JL. K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. Am J Pathol. 1993 Aug;143(2):545-54.
13. Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science. 1996 Jan 19;271(5247):350-3.
14. Morin-Papunen L, Rautio K, Ruokonen A, Hedberg P, Puukka M, Tapanainen JS. Metformin reduces serum C-reactive protein levels in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2003 Oct;88(10):4649-54.
15. Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, Schönbeck U, Libby P. Metformin inhibits proinflammatory responses and nuclear factor-kappaB in human vascular wall cells. Arterioscler Thromb Vasc Biol. 2006 Mar;26(3):611-7.
16. Sahra, I, Laurent K, Loubat A. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 2008;27;3576–3586.
17. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. BMJ. 2005 Jun 4;330(7503):1304-5.
18. Jiralerspong S, Palla SL, Giordano SH, Meric-Bernstam F, Liedtke C, Barnett CM, Hsu L, Hung MC, Hortobagyi GN, Gonzalez-Angulo AM. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. J Clin Oncol. 2009 Jul 10;27(20):3297-302.
19. Govindarajan R, Ratnasinghe L, Simmons DL, Siegel ER, Midathada MV, Kim L, Kim PJ, Owens RJ, Lang NP. Thiazolidinediones and the risk of lung, prostate, and colon cancer in patients with diabetes. J Clin Oncol. 2007 Apr 20;25(12):1476-81.
20. Currie CJ, Poole CD, Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. Diabetologia. 2009 Sep;52(9):1766-77.
21. Huang NL, Chiang SH, Hsueh CH, Liang YJ, Chen YJ, Lai LP. Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. Int J Cardiol. 2009 May 15;134(2):169-75.
22. Huang NL, Chiang SH, Hsueh CH, Liang YJ, Chen YJ, Lai LP. Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. Int J Cardiol. 2009 May 15;134(2):169-75.