

RESEARCH ARTICLE

Therapeutic Potential of an Anti-diabetic Drug, Metformin: Alteration of miRNA expression in Prostate Cancer Cells

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Abstract

Background and Aims: Prostate cancer (PC) is the most commonly diagnosed cancer in males in many populations. Metformin is the most widely used anti-diabetic drug in the world, and there is increasing evidence of a potential efficacy of this agent as an anti-cancer drug. Metformin inhibits the proliferation of a range of cancer cells including prostate, colon, breast, ovarian, and glioma lines. MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that downregulate gene expression. We aimed to evaluate the effects of metformin treatment on changes in miRNA expression in PC-3 cells, and possible associations with biological behaviour. **Materials and Methods:** Average cell viability and cytotoxic effects of metformin were investigated at 24 hour intervals for three days using the xCELLigence system. The IC₅₀ dose of metformin in the PC-3 cells was found to be 5 mM. RNA samples were used for analysis using custom multi-species microarrays containing 1209 probes covering 1221 human mature microRNAs present in miRBase 16.0 database. **Results:** Among the human miRNAs investigated by the arrays, 10 miRNAs were up-regulated and 12 miRNAs were down-regulated in the metformin-treated group as compared to the control group. In conclusion, expression changes in miRNAs of miR-146a, miR-100, miR-425, miR-193a-3p and, miR-106b in metformin-treated cells may be important. This study may emphasize a new role of metformin on the regulation of miRNAs in prostate cancer.

Keywords: Prostate cancer - miRNAs - metformin - miRNA profiling

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Introduction

Prostate cancer is the most common solid tumor in men followed by lung and colon cancer in the western world and second most common cause of cancer death among men (Jemal et al., 2009; Hernes et al., 2010). Also, prostate Current treatments for advanced PC are limited. Several novel drugs have been designed to target specific pathways involved in PC development and progression.

Metformin is the most widely used anti-diabetic drug in the world, but there is increasing evidence of a potential efficacy of this agent as an anticancer drug recently. Epidemiological studies show a decrease in cancer incidence in metformin treated patients. In addition, metformin decreases insulin resistance and indirectly reduces insulin level, a beneficial effect as insulin promotes cancer cell growth. Several reports also outline a direct inhibitory effect of metformin on cancer cell growth and an antitumoral action. Recently, numerous studies have shown that metformin decreases cancer cell viability and tumor growth (Murtola et al., 2008; Wright et al., 2009; Libby et al., 2009). The potential for application of metformin in oncology was

first recognized in retrospective epidemiological studies of diabetic patients with cancer. Several studies have reported decreased cancer incidence and cancer related mortality in diabetics receiving standard doses of metformin (1500-2250 mg/day in adults) (Decensi et al., 2010; Landman et al., 2010). Metformin also displays significant growth inhibitory effects on several cancer cells. In cell culture, metformin inhibits the proliferation of a range of cancer cells including prostate, colon, breast, ovarian, and glioma (Isakovic et al., 2007; Gotlieb et al., 2008).

MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that negatively regulate gene expression by mainly binding to 3' untranslated region (UTR) of target mRNAs at the post-transcriptional level. Recent studies have demonstrated that aberrant expressions of miRNAs are closely associated with the development, invasion, metastasis and prognosis of various cancers including PC. Regulations of the growth, differentiation and apoptosis of carcinoma cells controlled by the interaction of miRNAs with their target genes have been investigated recently (Iorio and Croce, 2009). The involvement of miRNAs in human PC has clearly demonstrated a correlation between miRNAs and their

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targets with prostate carcinogenesis in recent years (Shi, 2007; Jemal, 2009). Several miRNAs and their targets have been discovered to get expressed abnormally in PC, leading to the corresponding response in the development, invasion, and metastasis of this disease. The altered expressions of some selected miRNAs are useful as biomarkers for diagnosis, prognosis, and classification purposes of PC (Porkka, 2007; Mattie, 2006). Thus, understanding of the characteristic miRNA abnormalities, and restoring normal miRNA-mRNA regulation pathways could contribute to the development of novel therapeutic strategies in PC.

This study was undertaken to evaluate the effects of metformin treatment on the cellular miRNA expression changes in PC3 prostate cancer cell line, and possible association of miRNAs and metformin on prostate cancer has been analyzed for the first time. Results may reveal important roles of miRNAs through the regulation of different signaling pathways.

Materials and Methods

Cell line, culture conditions and drug

PC-3 (Androgen Independent Phenotype) prostate cancer cells were used as a model in this study which was obtained from ATCC. PC-3 cells were grown in RPMI-1640 Basal medium containing 2mM L-glutamine supplemented with 10% fetal bovine inactivated serum (FBS) and 1% penicillin/streptomycin in a standard cell culture incubator at 37°C, humidified 95% air, and 5% CO₂ atmosphere. Metformin AICAR (Toronto Research Chemicals, Inc.) was dissolved in culture media.

Real-time xCELLigence impedance analysis of the cytotoxicity of metformin on PC-3 cells

The real-time cell analyzer was used to evaluate cell survival, according to the supplier's instructions. After seeding 200 microL of the cell suspensions into the wells (10,000 cells/well) of the E-plate 96, cells were treated with increasing concentrations of metformin and monitored every 15 minutes for 190 hours.

Statistical analysis of cytotoxicity

Calculations of cytotoxicity were obtained by using the real-time cell analyzer integrated software, which performs a curve-fitting of selected "sigmoidal dose-response equation" to the experimental data points. Data are represented as mean (mmol/L) ± SD (n 5 5). For the statistical analyses of the proliferation experiments, one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests were used. The data are represented as means ± standard deviations. A value of $p < 0.05$ was considered to be statistically significant.

MicroRNA microarrays

RNA samples were used for analysis using custom multi-species microarrays containing 1209 probes covering 1221 human mature microRNAs present in miRBase 16.0 database. The array also contains 1037 probes covering 1052 mouse mature microRNAs, 679 probes covering 680 rat mature microRNAs and 484

probes covering 488 rhesus mature microRNAs. The sensitivity of the microarray is such that it could detect as low as 20 amoles of synthetic microRNA being hybridized along with each sample. The microarrays were produced by Microarrays Inc. (Huntsville, Alabama), and consisted of epoxide glass substrates that had been spotted in triplicate with each probe.

Sample processing

Quality of the total RNA samples was assessed using UV spectrophotometry and agarose gel electrophoresis. Low molecular weight (LMW) RNA (~0-200 nucleotides) was purified from total RNA by size fractionation on Nanosep 100K ultra-filtration columns (Pall Life Sciences, Ann Arbor, MI) and subsequent purification using the RNeasy MinElute Clean-Up Kit (Qiagen, Inc., Valencia, CA) using a small RNA Protocol. The LMW RNA samples were 3'-end labeled with Oyster-550 fluorescent dye using the Flash Tag RNA labeling Kit (Genisphere Inc., Hatfield, PA). Labeled LMW RNA samples were hybridized to the MicroRNA microarrays according to conditions recommended in the Flash Tag RNA labeling Kit manual. The microarrays were scanned on an Axon Genepix 4000B scanner, and data was extracted from images using GenePix V4.1 software.

Data pre-processing

Spot intensities were obtained for the 8816 features on each microarray by subtracting the median local background from the median local foreground for each spot. Detection Thresholds for each array were determined by calculating the 10% trim mean intensity of the negative controls spots and adding 5X the standard deviation of the background (non-spot area). The spot intensities and the Threshold (T) were transformed by taking the log (base 2) of each value. The normalization factor (N) for each microarray was determined by obtaining the 20% trim mean of the human probes intensities above threshold in all samples. The log₂-transformed spot intensities for all 8816 features were normalized, by subtracting N from each spot intensity, and scaled by adding the grand mean of N across all microarrays. The mean probe intensities for each of the 1209 human probes on each array were then determined by averaging the triplicate spot intensities. Spots flagged as poor quality during data extraction were omitted prior to averaging. The 1209 human non-control log₂-transformed, normalized, and averaged probe intensities were filtered to obtain a list of human microRNA probes showing probe intensity above T in at least 10% of the samples.

Microarray quality control

Each array contains probes targeting 11 different synthetic miRNAs, each of which is added at a mass of 200 amoles to each RNA sample prior to labeling and hybridization. Sensitivity of the microarray hybridization was confirmed by detection of hybridization signal for all 11 spikes well above the detection threshold. The array also contains a set of specificity control probes complementary to three different miRNAs. Each specificity control includes a perfect match, single

mismatch, double mismatch, and shuffled version of the probe. Specificity of the hybridization was confirmed by detection of hybridization signal on the microarray for the perfect match probes and not the double mismatch and shuffled version of the probes. Reproducibility of the arrays was determined by monitoring the hybridization intensity for the triplicate human spots on each array. The sensitivity, specificity, and reproducibility data for the arrays were compiled into a Quality Control report.

Differential expression analysis

For statistical analysis, samples were binned in to different treatment groups. The log₂-transformed and normalized spot intensities for the detectable human probes were examined for differences between the different groups of customer's interest using the ANOVA module built into the National Institute of Ageing (NIA) Array Analysis software. The statistical significance was determined using the False Discovery Rate (FDR) method which was proposed by Benjamini and Hochberg (1995). It is the proportion of false positives among all probes with P values lower or equal to the P value of the probes that is considered to be significant. It can also be viewed as an equivalent of a P-value in experiments with multiple hypotheses testing. FDR is an intermediate method between the P-value and Bonferroni correction (multiplying P-value by the total number of probes). The equation is: where r is the rank of a probe ordered by increasing P values, p_i is the P value for probe with rank i, and N is the total number of probes tested. FDR value increases monotonously with increasing P value.

Fold changes between different treatment and control samples were computed and presented along with the log₂ transformed spot intensities of the detectable probes. For Principal Component Analysis (PCA), samples were binned in to respective treatment groups. PCA was performed on the detectable probes using the module built in to the National Institute of Ageing (NIA) Array Analysis software (Sharov et al., 2005).

Hierarchical clustering of microRNA array data

Data for the detectable human probes were clustered using Cluster 3.0 software (De Hoon et al., 2004). Genes were median centered prior to hierarchical clustering. Hierarchical clustering was conducted using Centered Correlation as the similarity metric and Average Linkage as the clustering method. Intensity scale shown is arbitrary.

Results

Evaluation of the expression profiles of miRNAs were analysed in PC-3 cells treated with 1-5 mM doses of metformin at 24, 48 and 72 h time intervals. miRNA expression analysis was performed and expression rates were detected in a time and dose dependent manner. Metformin exerts its cytotoxic effects on PC-3 cells in a time- and dose-dependent fashion. The relative reduction in the cell number that evolved in cultures of PC3 cells was determined. These experiments aimed to assess the best concentration of metformin to be used in PC3 prostate cell line. A real-time cell analyzer (xCELLigence, Roche

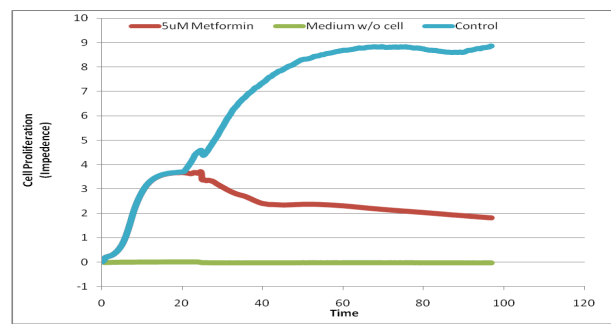


Figure 1. Proliferation of PC-3 Cells in Response to Metformin by X-celligence System

Table 1. miRNA Alteration in Response to Metformin (Fold change > 2-fold)

	miRNA Symbol	Change
Up-regulated:	hsa-miR-3198	3.05
	hsa-miR-206	2.89
	hsa-miR-3127	2.38
	hsa-miR-3620	2.35
	hsa-miR-494	2.3
	hsa-miR-3673	2.22
	hsa-miR-3149	2.11
	hsa-miR-4323	2.09
	hsa-miR-513a-5p	2.08
	hsa-miR-4299	2.04
Down-regulated:	hsa-miR-146a	0.36
	hsa-miR-1972	0.37
	hsa-miR-100	0.42
	hsa-miR-425*	0.43
	hsa-miR-193a-3p	0.45
	hsa-miR-93	0.45
	hsa-miR-181a	0.46
	hsa-miR-1280	0.47
	hsa-miR-26a	0.47
	hsa-miR-106b	0.47
	hsa-miR-1274a	0.48
	hsa-miR-937	0.5

Applied Science, Mannheim, Germany) was used to evaluate cell survival after 24, 48 and, 72 h exposure to metformin, and the results were shown in Figure 1. The IC₅₀ was found to be 5 mM revealed by Xcelligence system which was also confirmed by WST-1 assay.

miRNA expression profiles

Among the human miRNAs investigated by the arrays, 10 miRNAs were up-regulated and 12 miRNAs were down-regulated in metformin treated PC3 cells (Tables 1)

Discussion

Prostate cancer is the most frequently diagnosed cancer in men and the second most common cause of cancer related death in the western world. A number of in vitro and in vivo studies have shown an effect of metformin on inhibiting the growth of multiple cancer cell lines including that of prostate cancer. Ben Sahra et al. showed that metformin gave an up to 50% decrease in cell viability in human prostate cancer cell lines including PC-3 cells (Ben et al, 2008). The potential mechanism of activity of metformin has not been completely

understood. In an American population based case-control study, metformin usage was associated with a 44% risk reduction in prostate cancer incidence (Wright et al., 2009). Evidence for metformin on cancer cell growth has been clearly established in a series of in vitro studies. Actually, metformin exposes a strong and consistent anti-proliferative effect on several cancer cell lines including prostate, colon, pancreas, breast, and lung. Metformin is associated with a reduction of prostate cancer incidence, and also metformin inhibits human prostate cancer cells proliferation and tumor formation. At the cellular level, metformin interferes with cell cycle leading to G0/G1 or S phase arrest. Some studies suggested that metformin triggered a cell cycle arrest at the G0/G1 stage with a reduction in cyclin D1 levels in human prostate cancer cells in vitro (Ben et al., 2008; Wright et al., 2009).

Studies on the relationship between miRNAs and PC only started in recent years (Schaefer et al., 2009) but there is increasing evidence that altered expression of specific miRNAs are involved in the development and progression of prostate cancer (Baranwal and Alahari, 2010). As the progression of PC is rather a multiple combination with changes of expression of oncogenes and tumor suppressors, discovery of miRNAs goes a step forward to provide a novel regulatory mechanism for PC. Numerous studies are still being undertaken to clarify the complicated interaction between the multiple miRNAs and multiple target mRNAs. Many miRNAs have been found to be up-regulated in PC by miRNA expression profiling analysis. Overexpression of the oncomirs epigenetically silences the apoptosis related genes, and then induces tumor growth and metastasis. Loss of tumor suppressor miRNAs is another mechanism related to the progression of PC. Lin et al. analyzed eight down-regulated and three up-regulated miRNAs in PC tissues and cell lines, and found that miR-146a, one of the eight down-regulated miRNAs, was dramatically expressed. Forced expression of miR-146a resulted in remarkably reduced cell migration, invasion, proliferation, anti-apoptosis and metastasis in PC-3 cells, suggesting that miR-146a acts as a tumor suppressor in PC (Lin, 2008). The microRNA-200 family, which is down-regulated in PC, are a group of potential tumor suppressor miRNAs. PC-3 cells that have lost expression of miR-200c and miR-141, display a mesenchymal phenotype due to aberrant methylated CpG island near the miR-200c and miR-141, leading to invasion and metastasis of cancer cells (Kong et al., 2009; Vrba et al., 2010).

Our results showed that metformin treatment led to altered expression of some cellular miRNAs shown in tables. Our data suggests that expression changes in miRNAs of miR-146a, miR-100, miR-425, miR-193a-3p and miR-106b in metformin exposed cells may be important.

The preclinical and limited clinical evidence that is available coupled with the drug's favorable safety profile and low cost, justifies the need to pursue its further investigation in prostate cancer and other malignancies. Coupled with the implementation of new preclinical models, clinical trials will integrate to the development and effective use of metformin as a potential anticancer therapy. This work may demonstrate a novel role of

metformin on the regulation of miRNAs in prostate cancer.

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