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PPAR γ targeted oral cancer treatment and additional utility of genomics analytic techniques

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Abstract

Objective—Peroxisome proliferator-activated receptor γ (PPAR γ) agonists have been shown to have anti-proliferative, anti-angiogenic, and pro-apoptotic effects, leading to interest in their use as cancer therapeutics. Pioglitazone, an FDA approved type II diabetes medication and PPAR γ agonist, may have a role in adjuvant HNSCC treatment or prevention. Therefore, the purpose of this study was: 1) to treat oral cavity cancer cells with the PPAR γ activator, pioglitazone, to analyze gene expression changes and 2) to compare those changes with our pre-existing genomic data for development of hypothesis driven additional basic and clinical studies.

Study Design—Prospective *in vitro*

Methods—We utilized microarray technology, as well as, OCPlus and Ingenuity Pathway Analysis to analyze differential gene expression in tumor and pioglitazone-treated tumor cells on a genome wide level to demonstrate the feasibility of such an approach and determine appropriate sample size for future investigations.

Results—We found approximately 35 samples are required to adequately power future studies. We next discovered pioglitazone significantly affects iCOS-iCOSL and type II Diabetes Mellitus pathways as a putative anti-cancer mechanism.

Conclusions—Genome wide analysis is possible for exploration of differential pathway modulation and rapid hypothesis generation. Both inflammation and type II diabetes pathways were significantly altered and therefore might provide unique hypothesis-driven pharmacodynamic parameters for future *in vitro* or *in vivo* studies utilizing thiazolidinediones. These techniques could be applied to microarray or other high throughput data from a variety of hypothesis-

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generating research scenarios in otolaryngology (e.g. middle ear proteomics, sinus microbiome studies, etc.).

Level of Evidence—NA

Keywords

PPAR; thiazolidinedione; pioglitazone; head and neck cancer; genomics

Introduction

Head and neck squamous cell carcinoma (HNSCC) affects nearly 500,000 individuals worldwide each year, with nearly 55,000 patients in the United States. The 5-year survival rate is stable at approximately 50%, and additional therapeutics are needed to further improve survival. Peroxisome proliferator-activated receptor gamma (PPAR γ), initially investigated for its role in adipocyte differentiation, has anti-proliferative, pro-apoptotic and anti-angiogenic effects, leading to interest in the use of PPAR γ agonists for cancer treatment. The thiazolidinedione (TZD) drug class, FDA approved for type II diabetes, is potentially exploitable for aerodigestive chemoprevention and treatment.

Microarray data is increasingly utilized to determine pharmacologic targets for therapy, isolate pathways not previously implicated in certain processes, investigate biomarkers and gene expression profiles for progression and remission of cancer, classify patient subsets, and further characterize disease processes at the level of gene expression¹⁻³. We published one of the original head and neck cancer microarray studies analyzing differential gene expression in tumors versus normal samples and identified a tumorigenesis and recurrence profile⁴. We presently analyzed differential gene expression between pioglitazone-treated tumor cells and controls as a pilot study.

We had several additional goals with this project. First, we wanted to establish an appropriate “signal to noise” ratio for defining significant findings for the pioglitazone data to maximize true positives and minimize false positives for future studies⁵. To accomplish this goal, we utilized the freely available program, OCPlus. With OCPlus, algorithms allow the investigator to examine pilot data to predict future sample sizes and establish appropriate p-values for large data sets with multiple hypotheses⁶. In the OCPlus program, Pawitan *et al.* use a variant of Storey's method for this purpose. In this algorithm, the proportion of genes non-differentially expressed in a pilot set is designated p_0 . Therefore, $1-(p_0) \times 100$ is the percent of differentially expressed genes. Once this is known, the number of datasets required for an acceptably low False Discovery Rate (FDR) can be calculated. This allows an investigator to specify a statistically acceptable number of false positives and secondarily establish the sample size to reach this threshold^{6, 7}. This is vital in the initial stages of investigation when it is unknown which genes and pathways are relevant to the system in question. It also allows a circumstance to define a tolerable degree of inference⁸. Typical values considered acceptable for FDR in this method are significance levels ranging from 0.01 to 0.1, with 0.05 being frequently chosen^{9, 10}. The FDR has been successfully implemented in numerous previous microarray studies⁶. This would allow the researcher to

calculate whether five or fifty samples would be required to complete a scientifically valid high throughput analysis, and estimate project cost or labor.

Second, we were interested in both expression changes of individual genes and understanding the biological implications of such changes. A variety of bioinformatics applications focus on projecting gene data onto known metabolic and regulatory pathways through biological network analysis¹¹. We used Ingenuity Pathway Analysis (IPA) to map pioglitazone-induced changes in microarray data onto pre-existing metabolic pathways credentialed by the medical literature (Pub Med). Finally, we compared pioglitazone-induced changes with both tumors and normals from our previously published microarray data, to ascertain whether pioglitazone treatment could return the cells to a more normal squamous aerodigestive phenotype.

The reasoning/algorithm we devised for this analysis (Figure 1) could have potential application in other research areas in otolaryngology. For example, middle ear proteomic studies or sinus microbiome studies would generate large amounts of microarray data from limited numbers of human samples. Despite conditions including acute versus chronic infection, prior antibiotic use, etc., common features of either milieu could be discovered by carefully employing programs such as OCPlus and IPA. With this data, specific subsequent experiments could be designed to evaluate acute versus chronic infections, immune responses, etc.

Materials and Methods

Cell Line and Microarray

We utilized UMSCC9, an anterior tongue cancer cell line, for our analysis. UMSCC9 cells were treated with vehicle, DMSO, (three samples) or 4 μ M pioglitazone (three samples) for 72 hours. These cells were cultured at 5% CO₂, at 37°C, in MEM media containing 10% fetal calf serum (Life Technologies, Carlsbad, CA). Total RNA was isolated from both groups and cDNA was synthesized. cDNA from all groups was affixed to GeneChip Human Genome U133A Arrays (Affymetrix, Santa Clara, CA). In addition, previously published data from Ginos, *et al.* was utilized and therefore all interrogation of the genomic data was approved under the IRB for the published manuscript. This data was produced from cDNA of surgical specimens (41 HNSCC and 13 normal tissues) collected from patients who underwent surgery at the Department of Otolaryngology, University of Minnesota Hospital and Clinics hybridized to GeneChip Human Genome U133A Arrays. All techniques for the genomics experiment were exactly as previously published, as this analysis is a reinterrogation of this data set⁴. The treated cell line was experimentally prepared identically as the human tissues. The normal tissues for the array studies were supplied from healthy control subjects without a history of oral cancer, premalignant lesions, or periodontal disease from the buccal mucosa adjacent to the third molar.

Reporter gene experiments

Oral cancer Ca 9-22 cells were transiently transfected with a PPAR reporter gene and treated with 20 and 40 uM pioglitazone for 48 hours and reporter gene experiments were carried out as recently published. The experiment was repeated twice with 9 replicates per data point.¹²

Statistical Analysis of Genomic Data

Statistical analysis was performed using the OCPlus package and statistics software R (version 1.44.0)^{13,14}. Data was initially evaluated using the Empirical Operating Characteristics (EOC) function of OCPlus to calculate t-statistics (standard two sample t-stat with pooled variance), p-value, false discovery rate (FDR), and sensitivity for each gene. The EOC function also calculated p0, the percentage of genes not differentially expressed, using a variant of Storey's method. Using this derived p0, the data was further analyzed using a two-dimensional false discovery rate function in OCPlus, called fdr2d. This function generates the t-statistics, an auxiliary test statistic (the log of the standard error for each gene), and the local FDR for each gene using the two statistics generated. A graphic representation of the results with isobars for FDRs of 0.1, 0.2, and 0.3 was produced. Following analysis via fdr2d, data was further filtered by selecting cutoff values for acceptable levels of local FDR, the t-statistic, and the log(SE) to eliminate outlier values with deceptively low local FDR.

Ideal testing parameters were also calculated using the derived p0 from the EOC function. The OCPlus *samplesize* function was used to perform this task, which generated an FDR versus sample size curve on the basis of p0, D (a parameter for log2-fold change intensity), and crit, a defined critical value of DE (Differentially Expressed) genes. D was defined as ± 1 (equivalent to a fold-change of 0.5 and 2, respectively); crit was chosen as 0.01, in order to select the genes with 1% largest t-statistics as DE.

Pathway Selection and Analysis

Microarray data was then subjected to Ingenuity Pathway Analysis¹⁵. Data from the EOC computation with gene-specific p-values was interrogated. Out of 22,283 possible gene IDs, 21,009 genes were mapped by IPA with 12,940 genes being analysis-ready. Furthermore, this data was analyzed using IPA's Core Analysis tool, with inclusion of direct and indirect molecular relationships, utilizing all available data sources. Finally, pathways of interest were chosen using a B-H multiple testing correction p-value threshold of 0.05. Two pathways fulfilled these requirements and fold-change data from the original dataset was overlaid on the pathways of interest.

Results

Sample Size Determination

We first examined the level of stringency of our sample size for our microarray experiment. Data analysis using the Empirical Operating Characteristics (EOC) function of OCPlus yielded t-statistics, p-value, FDR, and sensitivity for each gene analyzed via microarray. Additionally, p0, the proportion of genes not differentially expressed, was calculated to be 0.98 using the EOC function. Figure 2A plots FDR versus t-statistic for the calculated p0 of

0.98. This plot shows the FDR is markedly elevated until the t-statistic is maximized. We next calculated the sample size necessary to achieve an acceptably low FDR. The derived p0 value was then used to analyze the microarray data to determine the FDR for each gene. The OCPlus program, fdr2d, produced FDR values for each gene as well as the t-statistic (log of the standard error for each gene), which is graphically represented in Figure 2B with FDR isobars of 0.1, 0.2, and 0.3 labeled. We calculated FDR for each gene so it could be plotted against sample size shown in Figure 2C. In this calculation, a FDR of 0 occurs with 35 samples. Therefore, about 70 Affymetrix U133A gene chip analyses of a cell line before and after pioglitazone treatment would be required to find all the differentially expressed genes on a U133A array.

Differential Gene Analysis

Next, genes were sorted based upon FDR and further filtered utilizing p value and t-statistic subroutines available in OCPlus. This multivariate analysis was undertaken to determine which genes were significantly modulated after pioglitazone treatment. The top five genes present and overlapping following multivariate analysis were selected for further investigation. Next, previously published data in which fold change of the five genes in normal and tumor samples from patients at the University of Minnesota was compared to fold change in tumor cell line following pioglitazone treatment and shown in Figure 3⁴. Of the genes identified, pioglitazone was found to modify gene expression only for GYG2 and ABHD2 with expression approaching that of normal tissue. GYG2, human glycogenin-2, is involved in glycogen biosynthesis and has not been described in human cancer biology or treatment¹⁶. ABHD2, Abhydrolase Domain Containing 2, is an androgen responsive gene recently shown to promote prostate cancer growth and resistance to chemotherapy. This gene may also be involved in colorectal carcinoma development^{17, 18}.

Next, data from EOC analysis (including gene-specific p-values) was interrogated. As stated in Methods, 21,009 genes were mapped using Ingenuity Pathway Analysis with 12,940 genes being analysis ready. The IPA Core Analysis tool was used to determine molecular relationships, with utilization of all available data sources. Pathways were selected using the B-H multiple testing correction with a p-value threshold of 0.05. The iCOS-iCOSL pathway in Th cells and type II Diabetes Mellitus (T2DM) pathway met these criteria and pertinent data was overlaid onto representations of the pathways.

The iCOS-iCOSL pathway is involved in T-cell activity including autoimmunity, and promotes regulatory T cells, an area of emerging importance in cancer immunity and immunologic therapy in mouse models of melanoma¹⁹. The T2DM pathway is involved in glucose homeostasis and a recent meta-analysis has suggested type II Diabetes Mellitus patients have an increased risk of colorectal cancer²⁰. Figure 4 depicts the outcome of IPA analysis and other pathways, which approached significance. A second biologically significant pathway analysis is shown in Figure 5. Supplemental Figures S1 and S2 show microarray data represented on diagrams of the iCOS-iCOSL and T2DM pathways, respectively.

To examine whether type 2 diabetes pathways could be functionally activated by pioglitazone we examined functional upregulation of a PPAR response element (PPRE)

common to many PPAR γ responsive genes. At 48 hours of pioglitazone treatment of oral cancer Ca-9-22 cells, we found a very profound up regulation of the PPRE ($p < 0.0001$), as judged by luciferase activation (Table 1).

Discussion

Our study aims were to determine pioglitazone effects on HNSCC canonical pathway signaling and to accurately calculate future microarray sample size with reasonable FDR and false negative rates. We also sought to identify novel hypothesis- driven biomarkers for pioglitazone cancer prevention interventions. IPA allowed us to filter individual gene data by p-value and investigate statistically significant, known metabolic and regulatory pathways via a Benjamini-Hochberg procedure. This determines FDR from individual p-values in multiple testing corrections (in a Bonferroni- or Storey-style adjustment). Ultimately, datasets with larger sample sizes produce higher yield; however, even smaller datasets may reveal statistically significant pathways. Furthermore, future studies adequately powered with a sample size of 35 may highlight additional pathways of interest.

Interestingly, pioglitazone appears to significantly modulate two of five genes affected: GYG2 and ABHD2 (Table 2). Pioglitazone reduces the expression of ABHD2, an androgen responsive gene, which promotes prostate and colorectal carcinoma^{17, 18}. GYG2 is involved in glycogen biosynthesis and although pioglitazone treatment increased GYG2 to near normal levels, the relevance of this gene to anti-tumor therapy is unclear¹⁶. Expectedly, not all genes differentially expressed between tumor and normal are “normalized” by pioglitazone and our small sample size may contribute to observed disparities. Also, comparing tissue samples with one cell line similarly may contribute to disparities.

The three other genes differentially expressed between tumor and normal cells were MLLT10, TRADD, and HTR5A. Pioglitazone did not return the expression of these genes to that of normal tissue. MLLT10 was the most significantly overexpressed gene after pioglitazone treatment. This gene encodes a protein that has a zinc finger region. Zinc finger protein motifs are found in a variety of proteins including transcription factors, nucleic acid trafficking, epithelial development, and cell adhesion²¹. MLLT10 is also known as AF 10 which undergoes rearrangement and can be important in leukemogenesis²². Recently, it has been shown to be associated with human female obesity²³. It has not been ascribed a function in oral cancer or as a biomarker of pioglitazone treatment. TRADD (Tumor necrosis factor receptor type 1-associated DEATH domain protein) was significantly decreased by pioglitazone administration (15-fold). This gene has been found to be a key adaptor molecule in the death receptor signaling pathway for apoptosis via NF κ B and MAP kinase pathways²⁴. Since it may be hypothesized that pioglitazone should increase TRADD expression, given its anti-tumor effects, the role of TRADD in HNSCC may be of future interest. Another gene found to be significantly downregulated was the HTR5A gene (12-fold following pioglitazone treatment). This gene has mostly been investigated in brain development and psychiatric disorders including bipolar affective disorder. However, it has been found to be highly upregulated at the mRNA level in breast cancer²⁵. The significance of HTR5A is similarly unclear at this time.

Upregulation of iCOS has been observed in a variety of tumor cells including melanoma²⁶. Furthermore, we noted a downregulation of IL-2 in our results and cytokines, such as IL-2, have been investigated as immunotherapy agents in HNSCC to induce tumor rejection and durable immune response²⁷. Multiple studies have shown IL-2 enhances cellular immune responses to tumors by stimulating the proliferation and activation of several types of leukocytes with antitumor activity, including natural killer cells, lymphokine-activated killer cells, antigen-specific T-helper cells, cytotoxic lymphocytes, macrophages, and B cells²⁸.

Classically, Th-1 mediated responses (IFN- α , GM-CSF, and IL-2) are more effective anti-tumor agents than Th-2 (IL-4, IL-6, IL-10); the former are often downregulated by tumor cells, and the latter upregulated²⁹. Specifically in HNSCC, T regulatory cells type 1 (Treg1), which mediate immune suppression, have been implicated as a cause of tumor progression. This occurs via expression of IL-10 and TGF- β in the local microenvironment, which are promoted at tumor sites and expand during disease progression and, interestingly, in patients with no ongoing evidence of disease who have undergone cancer therapy³⁰. Thus, our results are consistent with previous research and the suggestion that the iCOS-iCOSL pathway is a part of the mechanism of pioglitazone's anti-cancer effect is intriguing and provides data suggesting exploration of T cell subsets in tissues may be promising.

Other data supports previous pioglitazone research and figuratively act as internal controls. PPAR γ is upregulated with pioglitazone treatment in our data, shown in Supplemental Figure 2, as one would expect from a known PPAR γ agonist. Furthermore, TNF α is downregulated in studies in rheumatoid arthritis patients following pioglitazone administration as it was in our data³¹. Modulation of T2DM pathways is also expected, given pioglitazone's role in diabetes treatment. Glut4 is a molecule highly implicated in type II diabetes signaling, with a critical role in whole-body glucose homeostasis. We observed PPAR γ stimulation increasing Glut 4 expression, which has an unclear role in cancer biology³². By comparing the above genomic findings for multiple markers that are modulated with pioglitazone in the cell line, one can find that nuclear receptor, inflammatory mediator, and obesity findings are altered. With regard to the inflammation and obesity findings being linked to a potentially desirable therapeutic outcome other antidiabetic agents like metformin, are slated for a clinical prevention trial in oral cancer prevention (<https://clinicaltrials.gov/ct2/show/NCT02581137>). Additionally, the markers identified *in vitro* in the cell line could indeed become clinical trial biomarkers for any studies employing pioglitazone or other thiazolidinediones.

Previous studies have shown downregulation of NF κ B by pioglitazone in HNSCC, however, our analysis demonstrated potential upregulation^{31, 33}. This hints at the limitations of using a genomic mapping program without an adequate sample size; while individual pathways may be modulated in a statistically significant fashion, individual genes may not. In this study, if only genes with acceptable p or FDR values were selected, the false negative rate would have been prohibitively high, as illustrated in Figure 2A. Furthermore, CD36 (Figure 5) has been demonstrated to be increased in settings of defective insulin signaling, with thiazolidinediones promoting decreased expression of the molecule³⁴. In the current analysis, however, the molecule appears to be upregulated. Clearly molecule-specific changes are of limited utility with the current sample size.

We examined related literature to examine how OCPlus has been used in other settings. In one such paper, the fdr2d algorithm from OCPlus was used to describe an objective assessment of DE as a function of gene variability, thereby decreasing the rate of false positives.³⁵ The authors then utilized this statistic (also utilized in our study), to interrogate two microarray data sets, one from patients with BRCA1/2 mutations and the other from patients with diffuse large B-cell lymphoma. For BRCA data, fdr2d performed the same as traditional data analysis but was superior in the lymphoma data set, with fdr2d finding more genes that were regulated.³⁵ OCPlus was used on the same data sets to show that FDR and a mixture model (details non-contributory to this manuscript's scope), can be used for gene discovery, with careful explanation of their statistical approach outlined in their paper.³⁶

The techniques described in this paper could have additional applicability in high throughput studies in otolaryngology. For example, a recent proteomic study in middle ear effusions identified that a neutrophil mucosal response was predominant in a proteomic evaluation of 9 samples.³⁷ Using the data from the nine samples and applying the OC plus program could help the investigators to judge the degree of work and costs for definitive sample sizes to confirm and extend the findings for future grants or publications. The additional data from such a study could inform power analyses to determine number of samples required in analyzing the correlation a plethora independent variables could play in this condition. Illustratively, these data could be further be subjected to power analyses of smoking versus non-smoking households, age grouping, and bacterial population as examples. This type of recursive process, applied to high throughput data could form a powerful hypothesis-generating scenario for definitive advances. Applying Ingenuity Pathway Analysis to the neutrophil findings in any of the data sets would be further hypothesis generating, helping the investigators in elucidating canonical pathways in otitis media that could result in novel molecular therapeutic targets. These canonical pathways in otitis could differ from pediatric adenoiditis or sinusitis, for example. In other otolaryngology research, employing OC plus could predict scope of a project from a relatively small number of samples and minimal investment of time and financial resources.

Conclusions

Pioglitazone treatment of HNSCC cells modulates the iCOS-iCOSL and T2DM pathways, which may contribute to its observed anti-cancer effects. Using OCPlus and Ingenuity Pathway Analysis, we formulated a practical, yet sophisticated, method for pathway modulation following drug treatment and up to 35 microarray samples would adequately power similar future studies. Our technique would be useful for rapid hypothesis generation in many areas of otolaryngology translational science utilizing genomics, sequencing, or proteomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Analytic Process for Efficient Development of New Experiments and Clinical Trial Markers

Figure 1. Analytic process for efficient development of new experiments and clinical trial markers

The schematic representation of the approach used for result analysis used in this experiment is presented. This process lends itself to rapid result analysis and hypothesis generation for microarray experiments and may be useful in other areas of otolaryngology such as middle ear proteomics and sinus microbiome studies.

Figure 2.

A) Empirical Operating Characteristics (EOC) output from OCPlus. Associated t-statistics, p-value, false discovery rate (FDR), and sensitivity for each gene were obtained, and p0 was calculated. With a sample size of 6 in the setting of a p0 of 0.98, FDR is profoundly elevated until the t-statistic is maximized, at the expense of an unacceptably high false negative rate. **B) fdr2d Results for Microarray Data.** The results of fdr2d analysis are presented with FDR compared to t-statistic values. Isobars for 0.1, 0.2, and 0.3 FDR are labeled. **C) Samplesize function of OCPlus.** P0 derived from the EOC was used to generate an FDR vs. sample size, based upon a crit (a defined critical value of DE genes) of 0.01, which selects the top 1% largest t-statistics as differentially expressed, and D (a parameter adjusting for log2-fold change intensity), which restricts genes to those with fold-change of greater 0.5 and 2. D was chosen to be ± 1 equivalent to fold-change of 0.5 or 2, respectively.

Figure 3. Comparison to Genes from Previous U of MN Microarray

Data regarding five genes were extracted from a previous U of MN microarray analysis, in which gene expression in normal vs. tumor squamous cells was analyzed in an attempt to explicitly select genes present in normal cells that are altered in carcinoma and normalized by pioglitazone.

Figure 4. Canonical pathways with highest $-\log(\text{B-H p value})$ as selected by IPA analysis
Two pathways were statistically significant, with a B-H p value of 0.05 or greater: the iCOS-iCOSL signaling pathway in Th cells, and the Type II Diabetes Mellitus signaling pathway.

Figure 5. Representative signaling network alterations following administration of pioglitazone
A sample result following Ingenuity Pathway Analysis with microarray data mapped onto known pathways for rapid interpretation of the biological significance of experimental data. Protein-protein associations are indicated by edges containing single lines, whereas proteins that act upon another protein (controlling their expression) are indicated by arrows. Dotted lines represent indirect interactions. Proteins having higher expressions in pioglitazone-treated cells are shaded darker grey, those with lower expression are shaded lighter grey.

Table 1
Functional activation of T2DM pathways by pioglitazone

Pioglitazone treatment was applied to Ca-9-22 cells and functional up regulation of PPRE, common to many PPAR γ responsive genes, is shown via luciferase activation data.

Pioglitazone Concentration	Fold activation +/- SEM	P value
control	1.000 $\pm$ 0.04673 (N=9)	-----
20 uM	5.791 $\pm$ 0.1006 (N=9)	P < 0.0001
40 uM	3.966 $\pm$ 0.06259 (N=9)	P < 0.0001

Table 2
Top 5 genes whose expression was most affected by pioglitazone treatment

Following pioglitazone administration, all genes were analyzed using FDR and then filtered using p-value, logse, and tsat as described in Methods. The most modulated genes are represented in this table.

Gene	EOC			fdr2d		
	<i>tstat</i>	<i>pvalue</i>	<i>FDR</i>	<i>tstat</i>	<i>logse</i>	<i>fdr.local</i>
MLLT10	23.17	2.60E-05	0.30	23.17	2.36	0.05
GYG2	22.49	2.78E-05	0.30	22.49	2.59	0.06
ABHD2	-16.81	9.42E-05	0.68	-16.81	2.06	3.07E-06
TRADD	-15.86	1.32E-04	0.72	-15.86	3.74	3.07E-06
HTR5A	-12.31	3.35E-04	0.91	-12.31	0.35	1.57E-03