Drug repurposing and therapeutic anti-microRNA predictions for inhibition of oxidized low-density lipoprotein-induced vascular smooth muscle cell-associated diseases

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Drug repurposing is a new method for disease treatments, which accelerates the identification of new uses for existing drugs with minimal side effects for patients. MicroRNA-based therapeutics are a class of drugs that have been used in gene therapy following the FDA's approval of the first

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anti-sense therapy. This study examines the effects of oxLDL on vascular smooth muscle cells (VSMCs) and identifies potential drugs and anti-miRs for treating VSMC-associated diseases. The Connectivity Map (cMap) database is utilized to identify potential new uses of existing drugs. The success of the identifications was supported by MTT assay, clonogenic assay and clinical trial data. Specifically, 37 drugs, some of which are undergoing clinical trials, were identified. Three of the identified drugs exhibit IC₅₀ activities. Among the 37 drugs' targets, three differentially expressed genes (DEGs) are identified as drug targets by using both the DrugBank and the NCBI PubChem Compound databases. Also, one DEG, *DNMT1*, which is regulated by 17 miRNAs, where these miRNAs are potential targets for developing anti-miR-based miRNA therapy, is found.

Keywords: Cardiovascular disease; vascular smooth muscle cell; time-course microarray; oxidized low-density lipoprotein; differentially expressed gene; drug repurposing; microRNA; anti-miR; Gene Ontology enrichment analysis.

1. Introduction

Cardiovascular disease (CVD) is the most important cause of mortality worldwide. CVD includes any disease that affects the heart or circulatory vessels, including atherosclerosis (ATs), hypertension, coronary artery disease, myocardial infarction and peripheral vascular diseases. This disease originates in vascular lesions (such as abnormal vasomotor tone), and such lesions often have similar causes and are associated with similar disease processes.¹ According to the WHO report, CVD affects tens of millions of people each year. Therefore, a consensus concerning improving the diagnosis, treatment and prevention of CVD must be urgently established.²

The vessel wall is the integrative organ that comprises cells of three types, which are endothelial cells, vascular smooth muscle cells (VSMCs) and fibroblasts. It may self-modulate in response to various stimuli. Vascular abnormalities lead to phenotypic changes in the VSMCs, which constitute most of the walls of the blood vessel and have an important role in the pathogenesis of CVDs. Since CVDs are a very broad range of diseases, VSMC-associated diseases are used to specify diseases that are caused by phenotypic changes in the following discussion.

When the arterial vessel is subjected to risk factors (such as an oxidized low-density lipoprotein (oxLDL) or mechanical stress), which may initiate signaling pathways that are usually mediated by growth factors, triggering differential gene expression, and thereby inducing the dedifferentiation and phenotypic transition of VSMCs, the VSMC phenotype changes from a contractile state to a synthetic state and may promote the growth and migration of VSMC. Such a transition between different phenotypes is referred to as "phenotypic transition". It is a major cause of ATs,³ restenosis,⁴ hypertension,⁵ abnormal cell growth and apoptosis in VSMC.^{6,7}

ATs that is caused by VSMC proliferation is similar to cancer formation, in which tissue mass grows as a result of inflammation and uncontrolled cell proliferation. Several studies have suggested that the pathogenesis of both ATs and cancer is caused by malfunction of the same molecular pathways.^{8–10} Pathways that are common to ATs and cancer (such as PI3K/Akt) can mediate several functional and morphological alterations of VSMCs after they are activated, developing vascular diseases,¹¹ and

affecting the growth, apoptosis and regulation of the cycles of cells of various types, inducing cancer progression.¹² The MAPK pathway, which is involved in VSMC proliferation, hypertrophy and migration, and is critical to the pathogenesis of vascular diseases,¹³ has effects that are similar to the PI3K/Akt in the development of cancer.¹⁴

Based on the above observations, we hypothesize that some of the Food and Drug Administration (FDA)-approved cancer drugs may be repurposed for treating the VSMC-associated diseases. The repurposing of drugs may minimize side effects and accelerate drug development for cancer therapy.¹⁵ This work aims to identify drug molecules that may reverse VSMCs proliferation if the drugs reverse disease-induced signatures. The “signatures” means the “gene expression profiles” herein.

To identify such drugs, both up- and down-regulated differentially expressed genes (DEGs) were used to query the Connectivity Map¹⁶ (cMap) database. cMap, a web-based resource, is a robust tool that addresses queries by using five cell lines (MCF7, ssMCF7, PC3, HL60 and SKMEL). As stated by Lamb *et al.*,¹⁷ “The breast cancer epithelial cell line MCF7 has been extensively used as a reference in laboratories around the world. A subset of perturbagens was also profiled in the prostate cancer epithelial cell line PC3 and the non-epithelial cell lines HL60 (leukemia) and SKMEL (melanoma). This variety of cell types provides a chance to estimate the extent to which results are relied upon the experimental conditions for proper interpretation.” Even though meeting all conditions is impossible, the results obtained using various cell lines are acceptable to most of the researchers.

The dysregulation of microRNAs (miRNAs) expression causes many diseases, including cancer. MiRNAs may modulate gene expression and protein synthesis by negative regulation, by base pairing with the 3'-UTR of target mRNAs, which regulate many specific biological processes (BPs) (such as cell development, differentiation, signaling, metabolism and stress responses). Several specific miRNAs — miR-21,¹⁸ miR-143/145¹⁹ and miR-221/222²⁰ — have been identified as actively participating in the regulation of VSMC. In particular, miR-145 has been experimentally verified as having an anti-proliferative effect when over-expressed.²¹ The above results reveal that miRNAs have a critical role in the regulation of the VSMC phenotype transition and cause VSMC-associated diseases. Disease-associated miRNAs are a class of targets for developing miRNA-based therapies in which anti-sense oligonucleotides (anti-microRNAs anti-miRs) are used to inhibit miRNA expression.^{20,22}

MiRNA-based therapeutics are another class of drugs that are used in gene therapy. With the recent FDA approval of the first anti-sense therapy for treating hyperlipidemia, miRNA-based therapy has demonstrated its clinical effectiveness.²³ Recent progress in the pharmacological inhibition of disease-associated miRNAs offers great promise in developing novel miRNA-based therapeutics. For example, in three *in vivo* studies by Marquart *et al.*,²⁴ Najafi-Shoushtari *et al.*²⁵ and Rayner *et al.*,²⁶ anti-miR was used to probe the functions of miR-33 in cholesterol homeostasis. Those investigations demonstrated the inhibition of miR-33 by anti-miR-33, which elevates circulating high-density cholesterol levels, increases reverse cholesterol transport and reduces ATs. Even though the development of

Fig. 1. The concept for the treatment of oxLDL-induced VSMC-associated diseases. ‘Up’ and ‘down’ denote the up- and down-regulated DEGs, respectively. The dotted line denotes the interactive relationships among antimiR, miRNA and down-regulated DEGs. The arrows and the blunts denote activation and suppression, respectively.

antimiR-based miRNA therapeutics has had positive effects, it also has some side effects. The current design and use of antimiRs may have undesirable effects when antimiR-mediated is used to suppress miRNA expression, such as the down-regulation of RNA species other than the intended miRNA.

Figure 1 presents the concept for the treatment of VSMC-associated diseases. OxLDL induces both up- and down-regulated DEGs, which in turn trigger a VSMC phenotypic transition. Drugs could inhibit up-regulated DEGs or activate down-regulated DEGs, preventing VSMC phenotypic transition.

Aberrant over-expressed miRNA could silence specific target genes and thereby promote the VSMC phenotypic transition. AntimiR can be synthesized to silence the over-expressed miRNA, restore the down-regulated DEGs expression (dotted line in Fig. 1) to normal levels, stop the VSMC phenotypic transition, and prevent VSMC-associated diseases. On the other hand, miRNA can be introduced into the patient to target up-regulated DEGs, hence, inhibits the expression of up-regulated DEGs; restoring them to a normal level, and thereby suppress the VSMC phenotypic transition. This work examined the antimiR-mediated miRNA inhibition case.

The field of miRNA pharmacogenomics concerns the synergistic effects of miRNAs and the efficacy of drugs, which have potential clinical implications for personalized medicine.²⁷ For example, Shi *et al.*²⁸ reported a dual-drug delivery system that comprises miR-34a and paclitaxel has the potential for improving cancer therapy. Such a synergistic effect is not considered herein.

2. Methods

This study develops a strategy for identifying oxLDL-induced DEGs, potential drugs and therapeutic antimiRs based on microarray data. Figure 2 presents the workflow.

Fig. 2. Workflow of this study.

2.1. Datasets

To examine how oxLDL contributes to the response of VSMCs and to study its underlying signal transduction pathways, the microarray experiment GSE13139 from the Gene Expression Omnibus²⁹ was used. This experiment measured changes in gene expression that are activated by the binding of oxLDL to the *Lectin-like oxLDL receptor (LOX-1)* of endothelial cells at five time points of 0, 2, 6, 12 and 24 h. Each time point measurement was repeated twice to examine the temporal patterns of the gene expression in response to oxLDL. Each sample comprised three replicates that were prepared from HAECT cells.

2.2. Identification of DEGs and clustering analysis

In this work, *Bioconductor*³⁰ was utilized for identifying DEGs. First, the Robust Multi-Array Average (RMA) is used to normalize gene expression. Then, a model matrix is created (using the function, *model.matrix*) with rows and columns that denote the replicates (with information obtained at the five time points) and the time

points. Next, we seek a linear model that describes each probe/gene using the *lmFit* function that is provided in the *limma* package. Finally, the function, *cont.matrix*, is utilized to construct a contrast matrix, which enables a pairwise comparison of time points among the three replicates (using the function *contrast.fit*).

Many statistical methods exist for identifying DEGs, of which the significance analysis of microarrays (SAM),^{31,32} the empirical Bayes analysis of microarrays (EBAM),^{33,34} and the empirical Bayes statistics (eBayes)³⁵ are commonly used. In a previous study,³⁶ we found that eBayes, SAM and EBAM identified cancer genes with similar accuracy of around 20%. Therefore, eBayes is used herein to identify top-ranked DEGs with assumed adjusted *p*-values, p_{eBayes} , of less than 5%. The eBayes algorithm overcomes the possibility of identifying genes with small fold-changes that may be statistically significant.

The eBayes algorithm³⁷ computes moderated *t*-statistics, moderated *F*-statistics, and log-odds of differential expression by the empirical Bayes shrinkage of the standard errors towards a common value. The moderated *t*-statistic is defined by

$$\tilde{t}_{pq} = \frac{\hat{B}_{pq}}{\tilde{s}_p \sqrt{C_{pq}}}, \quad (1)$$

where *p* and *q* are the *p*th gene and the *q*th time, respectively and the contrast estimator $\hat{B}_{pq}$ denotes the difference between two classes. $\tilde{s}_p$ is the estimated shrinkage of the standard deviation of the *p*th gene expression level, and C_{pq} is the *p*th diagonal element of the covariance matrix.

In this study, we focus on genes that are consecutively up- or consecutively down-regulated in four time intervals (0 versus 2 h, 2 versus 6 h, 6 versus 12 h and 12 versus 24 h), because these genes are unambiguously up- or down-regulated. We define these as the DEGs. We hypothesize that a drug molecule in cMap may potentially reverse the disease signature if the drug-induced gene expression profile is significantly negatively correlated with the disease-induced signature. Accordingly, given two sets of disease-induced DEGs, i.e. both up- and down-regulated DEGs, anti-correlated drug-induced signatures in cMap were sought.

Furthermore, we made use of both up- and down-regulated clusters to identify the drugs. It is because genes belong to the same cluster are typically involved in similar BPs. A graph clustering algorithm called “clustering with overlapping neighborhood expansion” (ClusterONE)³⁸ is used to generate both up- and down-regulated clusters. ClusterONE deals with weighted graphs and yield overlapping clusters for predicting potentially protein complexes in protein–protein interaction (PPI) networks. The parameters provided by ClusterONE are grouped into two modes — i.e. basic (minimum size, minimum density, edge weights) and advanced (node penalty, haircut threshold, merging method, similarity, overlap threshold, seeding method). Default values are used in the present study, i.e. basic (3, auto, unweighted) and advanced (2, 0, single-pass, match coefficient, 0.8, from unused nodes).

The procedure for identifying clusters is described as follows: first, given both up- and down-regulated DEGs and the PPI information from BioGRID,³⁹ the up-regulated (down-regulated) PPI network is extracted as the maximal subnetwork from BioGRID consisting entirely of up-regulated (down-regulated) genes. Second, ClusterOne was run on the up-regulated (down-regulated) PPI network to generate up-regulated (down-regulated) clusters. Only clusters with p -values, called $p_{\text{ClusterONE}}$ -values, less than 0.05 were kept, and isolated DEGs were removed. Third, both up- and down-regulated clusters were combined in pairs to query cMap.

2.3. Gene ontology enrichment analysis

DAVID⁴⁰ is a web-based resource, which accepts a gene list and performs Gene Ontology (GO) BP⁴¹ and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway⁴² term enrichment analysis to identify the BPs and pathways that are more strongly associated with the given gene list. The list of DEGs was submitted to DAVID to obtain enriched BPs and pathways.

2.4. Kolmogorov–Smirnov test

The set of consecutively up- and down-regulated DEGs is conjectured possibly to cluster among the top-ranked DEGs, such that up- and down-regulated DEGs tend to have smaller p_{eBayes} -values. The Kolmogorov–Smirnov test (KS-test) is a distribution-free test that compares the cumulative distributions of two sets of data. It was used herein to seek the difference between two distributions: (i) the consecutively up- and down-regulated DEGs and (ii) the non-consecutively up- and down-regulated DEGs. The top-ranked DEGs were divided into 10 groups by using three different ways of partitions for the KS-test. DEGs were sorted in order of ascending p_{eBayes} -value. Started with a total of 7245 DEGs, we examined the following three cases of partitions: (a) using a constant ratio of 0.6, then the grouping started from 7245 down to 73, (b) subtraction using a constant difference of 800, then the grouping started from 7245 down to 45 and (c) subtraction using multiple constant differences, then the grouping started from 7245 down to 125.

2.5. Drug repurposing — IC50 measurements

The drug-gene interaction database, cMap,¹⁶ is utilized herein to find potential drugs for treating VSMC-associated diseases. CMap drugs' information (cMap name, p_{cMap} -value, enrichment score (ES), connectivity score (CS), etc.) are returned after query. Both ES and CS lie between -1 and $+1$.

According to the cMap webpage (http://portals.broadinstitute.org/cmap/help-topics_frames.jsp), a negative/positive ES or CS indicates that the perturbation tended to reverse/induce the expression of the query signature.^{16,17} Namely, a drug with a lower negative ES or CS indicates that it is more likely to be a potential drug.

After processing the DEGs without/with using ClusterONE, both sets of up- and down-regulated DEGs/clusters are used to query cMap. Potentially repurposed drugs with p -value, p_{cMap} , of less than 0.05, and both ES and CS of less than 0 are identified. The results of a few drug identifications are supported by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay or clonogenic assay data. The accuracy of identification of drugs are decided according to IC50 measurements.

Cell viability (the IC50 values) was determined by performing the MTT and clonogenic assay. Relevant protocols have been described in our earlier works.^{15,43} Two non-small cell lung cancer cell lines, A549 and H460, were used in both the MTT and clonogenic assay. For the MTT test, cells were seeded in a 96-well microplate for 16–20 h and treated with the indicated drugs. After drug treatment for 72 h, 50 μL MTT solution (2 mg/mL) per well was added and incubated at 37°C. Two hours later, 150 μL liquid per well was removed; DMSO was added, and the absorbance at 570 nm was detected using an ELISA reader (Infinite® M1000, TECAN, Switzerland). The untreated groups were regarded as 100% viable.

For the clonogenic assay, cells were seeded in six-well plates at 500 cells/well for 7–10 days. Each well contained 1.5 mL RPMI medium for culturing and tested drugs were added 24 h after seeding with the cells. The medium and drugs were changed once on day four. Following treatment, the cells were washed with PBS, and the colonies were fixed (acetic acid: methanol, 1:3) and stained with 0.5% crystal violet in methanol. After removing the excess crystal violet and rinsing with tap water, the colonies were counted manually.

Furthermore, the “ClinicalTrials.gov results database” (<https://clinicaltrials.gov/>) was used to find drugs that are undergoing clinical trials.

2.6. Identify drug-targeted DEGs, miRNAs and anti-miRs

Drug targets were obtained by submitting potential drugs to two independent databases — DrugBank⁴⁴ and NCBI PubChem Compound,⁴⁵ and the results were compared with both consecutively up- and down-regulated DEGs to identify drug-targeted DEGs.

In this work, two miRNA target gene resources — miRTarBase (version 6.0)⁴⁶ and TarBase (version 7.0)⁴⁷ — are used to identify miRNAs that hybridize drug-targeted DEGs, the miRNA-target regulatory relations are obtained, and these miRNAs may be silenced or inhibited using anti-miRs. MiRTarBase has accumulated many miRNA-target interactions which are validated experimentally and collected by reporter assay, western blot, microarray and sequencing experiments.⁴⁶ TarBase provides high quality experimentally validated miRNA:gene interactions, and utilized detailed meta-data to enhance the confidence of these interactions.⁴⁷

The set of overlapped drug-targeted DEGs and miRNAs are shown in grey in the bottom two boxes in Fig. 2. Over-expressed miRNAs may down-regulate certain

genes, inducing VSMC phenotypic transition. Certain drugs may target those down-regulated DEGs and restore their expression levels.

As described in the introduction, anti-miR-based miRNA therapeutics may be useful in inhibiting the aberrant expression of miRNA as anti-miRs can be used to target over-expressed miRNAs and silence their expression, inhibiting phenotypic transitions. Anti-miRs are molecules that have potential in treating VSMC-associated diseases. Accordingly, anti-miRs that can hybridize the group of miRNAs that target down-regulated DEGs are identified. The silencing of the miRNAs enables the expression of down-regulated DEGs to be restored; therefore, these anti-miRs have potential in developing anti-miR-based miRNA therapy.

3. Results

3.1. DEGs and their related functional terms

A total of 7245 DEGs were identified by eBayes analysis; these DEGs were compared with a CVD-associated gene list that was established by assembling information from SABiosciences (QIAGEN company)^{48,49} and a literature review. At least 86 genes are relevant to the formation of CVDs. Among these 86 genes, 48 are associated with the proliferation of VSMCs. As indicated in Table 1, these 48 DEGs are labeled with the following functional terms; “response to stress”, “apoptosis”, “blood coagulation and circulation”, “adhesion molecules”, “extracellular molecules”, “lipid transport and metabolism”, “cell growth and proliferation” and “transcription regulators.”⁴⁸

A website has been established to record our findings, which can be accessed at <http://ppi.bioinfo.asia.edu.tw/vsmc/> by clicking the “OxLDL-induced” hyperlink. It provides (i) lists of CVD-associated genes that were compiled from SABiosciences and a literature review and (ii) the 48 VSMC proliferation-associated DEGs along with their references.

Table 1. The 48 DEGs of the VSMC-associated diseases, and their functional terms.

Gene name (functional terms)					
<i>ABCA1</i> (6)	<i>CFLAR</i> (2)	<i>FGF2</i> (5, 7)	<i>IL6</i> (1)	<i>MMP2</i> (5)	<i>SERPINE1</i> (3, 5)
<i>BCL2A1</i> (2)	<i>COX1</i> (1, 6, 7)	<i>FN1</i> (1, 4, 5)	<i>ITGA2</i> (3, 4)	<i>NFKB1</i> (1, 2, 8)	<i>SOD1</i> (1)
<i>BIRC3</i> (2)	<i>CSF1</i> (7)	<i>HBEGF</i> (5, 7)	<i>ITGA5</i> (4)	<i>NPY</i> (3, 5, 7)	<i>TGFB1</i> (2, 7)
<i>CAT</i> (2, 7)	<i>CSF2</i> (1, 5, 7)	<i>ICAM1</i> (4)	<i>KLF2</i> (8)	<i>PDGFA</i> (3, 5, 7)	<i>TGFB2</i> (7)
<i>CCL2</i> (1, 2, 4, 5)	<i>CTGF</i> (1, 4, 5, 7)	<i>IFNAR2</i> (1, 5)	<i>LDLR</i> (6)	<i>PDGFB</i> (1, 3, 5, 7)	<i>TIMP2</i> (7)
<i>CCL5</i> (1, 4, 5)	<i>CXCL1</i> (1)	<i>IL1A</i> (2, 5, 7)	<i>LIF</i> (5, 7)	<i>PLIN2</i> (6)	<i>TNFAIP3</i> (2, 8)
<i>CD44</i> (4)	<i>EGR1</i> (8)	<i>IL1B</i> (4)	<i>MMP1</i> (5)	<i>PPARA</i> (6, 8)	<i>VEGFA</i> (1, 4, 5, 7)
<i>CDH5</i> (4)	<i>ENG</i> (3, 4)	<i>IL1R1</i> (1)	<i>MMP14</i> (5)	<i>PPARG</i> (1, 6, 8)	<i>VWF</i> (1, 3, 4, 5)

Notes: Numbers ‘1’ to ‘8’ denote ‘(1) response to stress’, ‘(2) apoptosis’, ‘(3) blood coagulation and circulation’, ‘(4) adhesion molecules’, ‘(5) extracellular molecules’, ‘(6) lipid transport and metabolism’, ‘(7) cell growth and proliferation’ and ‘(8) transcription regulators’, respectively.

3.2. GO enrichment analysis

DAVID can only process a limited number of input genes. Therefore, the top 3000 DEGs (with adjusted p -values, p_{eBayes} , of less than $5.48\text{e-}03$) were submitted to DAVID for clustering, yielding enriched BP- and pathway-related terms.

An enrichment study of the following two sets of genes was also conducted.

- (1) A total of 448 consecutively up- and 452 consecutively down-regulated DEGs that were filtered from the top 7245 DEGs, and
- (2) A total of 283 consecutively up- and 278 consecutively down-regulated DEGs that were selected from the top 3000 DEGs.

Only BP or pathway terms with a p -value, p_{GO} , of less than 0.05 are retained. These enriched BPs or pathways are associated with cancer- or cell regulation-related events. Our results demonstrate that many DEGs participate in the cell proliferation or apoptosis regulatory pathways, which result in the VSMC-associated diseases or cancer.

Details of the top two clusters of information about enriched BPs and pathways can be found at <http://ppi.bioinfo.asia.edu.tw/vsmc/> by clicking the “OxLDL-induced” hyperlink. This website provides important genetic information. Including chromosomal locations, Unigene ID, pathways, and adjusted p_{eBayes} -values.

3.3. KS test

To perform the KS-test, 7245 DEGs ($p_{\text{eBayes}} < 0.05$) were sorted in order of ascending p_{eBayes} -value, which were divided into ten groups via using three different ways of partitions (in Sec. 2.4). The maximum difference (D) between the cumulative distributions of (i) the consecutively up- and down-regulated DEGs and (ii) the non-consecutively up- and down-regulated DEGs, for the three cases were determined. The D values for the three cases studies are given by: $D_a = 0.3$, $D_b = 0.3$ and $D_c = 0.4$, with a corresponding p -value, p_{KS} , of 0.787, 0.787 and 0.418, respectively. These results do not suggest that the consecutively up- and down-regulated DEGs tend to cluster within the top-ranked DEGs. Details of the KS-test results and comparative plots can be found at <http://ppi.bioinfo.asia.edu.tw/vsmc/> by clicking the “OxLDL-induced” hyperlink.

3.4. Potential repurposed drugs for VSMC-associated diseases

Started with the 448 up- and 452 down-regulated DEGs, a total of 298 up- and 322 down-regulated DEGs were retained for analysis, because certain DEGs are not recorded by cMap. 37 drugs with both ES and CS of less than 0 were identified (p_{cMap} -value < 0.05). These drugs have the potential to be repurposed to inhibit the VSMC-associated diseases. Among these drugs, three (8.1%) — GW-8510, 8-aza-guanine and camptothecin — had experimentally determined IC50 activities, but

Table 2. The list of identified potential drugs for the VSMC-associated diseases.

Drug name	Potential drugs from cMap				IC50 activities			
	ES	<i>p</i> -value	Cell line	CS	MTT		Clonogenic	
Alsterpaullone	−0.998	0.00004	PC3	−0.928				
Spaglumic acid	−0.992	0.00022	HL60	−0.778				
Lidocaine	−0.989	0.00026	PC3	−0.895				
Camptothecin	−0.981	0.00082	MCF7	−0.764	O	< 1	—	
H-7	−0.977	0.00107	PC3	−0.719				
CP-944629	−0.975	0.00129	PC3	−0.78				
Disopyramide	−0.968	0.00229	MCF7	−0.699				
0179445-0000	−0.788	0.0041	MCF7	−0.545				
Tiletamine	−0.948	0.00588	MCF7	−0.6				
Famotidine	−0.939	0.00767	PC3	−0.753				
Baclofen	−0.937	0.00821	PC3	−0.803				
Demeclocycline	−0.935	0.00891	PC3	−0.524				
Scopolamine	−0.931	0.00984	MCF7	−0.627				
Tolbutamide	−0.734	0.01005	MCF7	−0.708				
Netilmicin	−0.725	0.01166	PC3	−0.743				
Guaifenesin	−0.925	0.01171	PC3	−0.534				
Neomycin	−0.918	0.01352	PC3	−0.82				
Fluocinonide	−0.918	0.01354	PC3	−0.69				
Bromopride	−0.917	0.01416	PC3	−0.575				
Tolfenamic acid	−0.915	0.01453	PC3	−0.724				
Flumetasone	−0.914	0.01497	PC3	−0.655				
GW-8510	−0.913	0.01529	PC3	−0.855	O	> 5	—	
Etomidate	−0.906	0.01795	MCF7	−0.612				
Sulfamethoxazole	−0.904	0.01845	PC3	−0.688				
Flucytosine	−0.692	0.01936	PC3	−0.651				
Hydroxyachillin	−0.895	0.02205	MCF7	−0.538				
Moracizine	−0.682	0.0222	MCF7	−0.641				
Fenbufen	−0.892	0.02322	PC3	−0.678				
Edrophonium chloride	−0.892	0.02342	PC3	−0.907				
Suprofen	−0.882	0.02827	MCF7	−0.64				
Cinnarizine	−0.88	0.02897	MCF7	−0.634				
Bucladesine	−0.651	0.03503	MCF7	−0.625				
Bupropion	−0.86	0.03891	MCF7	−0.552				
8-azaguanine	−0.86	0.03899	MCF7	−0.67	O	> 5	O	null
Prestwick-675	−0.641	0.03991	MCF7	−0.667				
Ethotoin	−0.857	0.04066	PC3	−0.645				
Sisomicin	−0.849	0.04587	MCF7	−0.523				

Notes: ‘ES’ denotes enrichment score, ‘CS’ denotes connective score. *Italics* fonts denote drugs under clinical trial. ‘-’ denotes not available, and ‘O’ denotes one of the following measurements: MTT assay, clonogenic assay or both.

none is undergoing clinical trials. Table 2 presents the cMap and IC50 information for these 37 drugs. Among them, ten (27%) are under clinical trials for CVD-associated diseases. Table 3 summarizes the clinical trial identifiers and information about the clinical studies. These 10 drugs have higher potential to be repurposed than others to inhibit the VSMC-associated diseases.

Table 3. cMap drugs with their clinical studies information.

Drug name	Clinical trials identifier	Related diseases
Lidocaine	NCT01742845	Carotid ATs
H-7	NCT00570752	ATs
Disopyramide	NCT00000556/NCT02294955/NCT00589303	CVDs, HD, AF/AF/AF, HF
Famotidine	NCT00683111, NCT01062516/NCT00950339	Acute CS, Acute MI/CHD
Scopolamine	NCT01553513/NCT02479581	CAD/Valvular HD
Neomycin	NCT01097811	Hypertension
Etomidate	NCT01495949/NCT01248234/NCT00415701	Valvular HD, CAD/Hypertension/CAD
Bupropion	NCT02638129/NCT00689611/NCT00181818	CVDs/MI/CVDs, MI
Guaifenesin	NCT00005704/NCT00680927	CVDs, HD/AF
Moracizine	NCT00000556/NCT00000526/NCT00000504	CVDs, HD, AF/CVDs, HD, MI, CD/CVDs, HD

Notes: ATs, atherosclerosis; CVDs, cardiovascular diseases; HD, heart diseases; AF, atrial fibrillation; HF, heart failure; CS, coronary syndrome; MI, myocardial infarction; CHD, coronary heart disease; CAD, coronary artery disease; CD, coronary diseases.

3.5. Drugs identified by clustering analysis

The use of ClusterONE algorithm produced only one up-regulated cluster with a size of three ($p_{\text{ClusterONE-value}} < 0.03$), as well as two down-regulated clusters with sizes of six ($p_{\text{ClusterONE-value}} < 0.002$) and three ($p_{\text{ClusterONE-value}} < 0.03$). As there were so few predicted clusters, we relaxed the threshold to accept another down-regulated cluster of three ($p_{\text{ClusterONE-value}} < 0.08$).

It was found that taking the union of the up-regulated cluster and the down-regulated cluster with $p_{\text{ClusterONE-value}} < 0.03$, it yielded the highest IC50 hit ratio, i.e. 22.2% (8/36). In other words, cMap identified 36 drugs, of which eight were verified as effective by performing IC50 experiments. Combining the up-regulated cluster with the down-regulated cluster with $p_{\text{ClusterONE-value}} < 0.08$ yielded the second highest hit ratio of 14.3% (6/42). Finally, combining the up-regulated cluster with the down-regulated cluster of size six yielded the lowest hit ratio of 12.5% (2/16).

Notably, the hit ratios for the above three combinations are at least 12.5%, exceeding the value of 8.1% that is obtained without using ClusterONE. This result suggests that the use of gene clusters gives a better hit ratio. Among the predicted drugs, one drug, 8-azaguanine was identified three times and two drugs, i.e. camptothecin and GW-8510, were identified twice in the three combinations, and they were also identified without using ClusterONE. In addition, three drugs, i.e. thio-guanosine, verteporfin and azacitidine, were identified twice using ClusterONE. Interesting, there are three drugs, i.e. camptothecin, mitoxantrone and vorinostat, that have a favorable IC50 in the MTT assay ($< 1 \mu\text{M}$).

3.6. Drug-targeted DEGs identification

A total of 37 drugs were submitted to two independent drug databases — DrugBank and NCBI PubChem Compound — to identify their targets which were used to

Table 4. The results of identified drug-targeted DEGs.

cMap drug	Drug-targeted DEGs from DrugBank	Drug-targeted DEGs from NCBI PubChem compound
Cinnarizine	<i>HRH1</i> (up)	<i>HRH1</i> (up)
Flucytosine	<i>DNMT1</i> (down)	<i>Not available</i>
Lidocaine	<i>SCN9A</i> (up)	<i>SCN9A</i> (up)

Notes: 'Up' and 'down' denote the up- and down-regulated DEGs, respectively.

match the up- and down-regulated DEGs. The DEGs *HRH1* and *DNMT1* are targeted by cinnarizine and flucytosine, respectively. Also, *HRH1* is a drug-targeted DEG that is found in both databases. One other DEG, *SCN9A*, which was obtained from both DrugBank and NCBI PubChem Compound, was identified as a target of lidocaine. *DNMT1* is a down-regulated DEG, whereas, both *HRH1* and *SCN9A* are up-regulated DEGs.

Histamine and its receptors, including *HRH1*, have an important role in promoting the formation of atherosclerotic lesions by increasing vascular permeability to low-density lipoprotein (LDL), and their signaling pathways can induce the expression of downstream genes, which are involved in ATs.⁵⁰ Histamine may inhibit cardiomyocyte apoptosis and increase macrophage vitality, reducing the risk of death in the course of acute myocardial infarction.⁵¹ Up-regulated *DNMT1* activity accelerated the formation of foam cells, and the over-expression of *DNMT1* is a significant indication of inflammation, advanced human plaques.⁵² In several types of the smooth muscle cell, the expression of *SCN9A* may result in cell migration, and silencing *SCN9A* may suppress cell migration and prevent metastasis.⁵³ Table 4 summarizes the identified drug-targeted DEGs.

The up- and down-regulated DEG clusters that were obtained by ClusterONE analysis were also used to query cMap. Then those identified drugs were also submitted to DrugBank and NCBI PubChem Compound to identify drug targets. No drug-targeted DEG was found, mainly because the up- and down-regulated clusters include very few DEGs.

3.7. MicroRNA-target interactions

MiRNAs can mediate post-transcriptional gene silencing, repress gene expression. Numerous miRNAs which have been identified as novel biomarkers and potential therapeutic targets in treating VSMC-associated diseases or cancer.

The miRNA-target regulatory relations of the three drug-targeted DEGs, *HRH1*, *DNMT1* and *SCN9A*, are obtained by searching against the miRTarBase and TarBase databases. Table 5 summarizes the related results of identified miRNAs, their target genes, expression status, the cause of disorders and references. These predicted miRNAs may target these drug-targeted DEGs, resulting in gene silencing or degradation, promoting the VSMC-associated diseases.

Table 5. List of drug-targeted DEGs and their identified miRNAs, associated CVDs and expression status.

Drug-targeted DEG	Mature miRNA	miRNA-associated diseases	miRNA-associated dysregulation
<i>HRH1</i>	miR-124-3p	Stroke	Up-regulated ⁵⁸
	miR-192-5p	AF	Up-regulated ⁵⁹
<i>DNMT1</i>	miR-100-5p	ATs	Up-regulated⁶⁰
	miR-126-3p	ATs	Down-regulated ⁶¹
	miR-140-5p	Stroke	Up-regulated ⁶²
	miR-148a-3p, 218-5p	HF	Up-regulated⁶³
	miR-148b-3p	HF	Down-regulated ⁶⁴
	miR-149-5p	CAD, MI	Down-regulated ^{65,66}
	miR-155-5p	AF, ATs	Up-regulated ^{67,68}
	miR-185-5p	CH	Down-regulated ⁶⁹
	miR-193b-3p	AF	Up-regulated ⁶⁷
	miR-26a-5p	MI	Up-regulated ⁷⁰
	miR-29b-3p	HF	Up-regulated ⁷¹
	miR-30b-5p	CH, HF	Down-regulated ^{72,73}
	miR-342-3p	HF	Down-regulated ⁷³
	miR-484	CAD	Down-regulated ⁷⁴
	miR-877-3p, 1260b	Not available	Not available
<i>SCN9A</i>	miR-335-5p		

Notes: AF, atrial fibrillation; ATs, atherosclerosis; HF, heart failure; CAD, coronary artery disease; MI, myocardial infarction; CH, cardiac hypertrophy.

Based on Table 5, miR-126 may suppress the expression of a vascular cell adhesion molecule 1 (*VCAM-1*) and decrease vascular inflammation.⁵⁴ MiR-155 may prevent the development and progress of ATs, and may participate in the inflammatory response and MAPK pathway by targeting *MAP3K10*.⁵⁵ The down-regulation of miR-193b induces the expression of the urokinase-type plasminogen activator (*uPA*), which increases the number of VSMCs, resulting in proliferative vasculopathy with intimal hyperplasia.⁵⁶ OxLDL down-regulated the expression of *DNMT3b* in the primary human aortic smooth muscle cells (HASMCs) by up-regulating miR-29b, and regulated *MMP-2/MMP-9* genes that are involved in cell migration, suggesting that miRNA-mediated regulation is crucial in ATs.⁵⁷

With respect to miRNA-target interactions, miRNA-regulated DEGs can be classified into three types of modules, which are one-to-one (*SCN9A*), one-to-many (*DNMT1* and *HRH1*), and many-to-many (not found herein). In this work, a total of three miRNA-regulated modules are identified and shown in Fig. 3.

3.8. MicroRNA target candidates for antimiRs

From Table 5, it was found that the drug-targeted DEG, *DNMT1*, is targeted by 17 miRNAs. These miRNAs are potential candidates used in developing antimiRs in miRNA inhibition for VSMC-associated diseases. Among these 17 miRNAs, eight (47%) exhibit up-regulated expressions (boldface words in Table 5), seven (41%)

Fig. 3. The miRNA-regulated modules for the drug-targeted DEGs. (a) The one-to-one module, (b) and (c), the one-to-many module. Both *HRH1* and *SCN9A* genes belong to the drug-targeted up-regulated DEGs; whereas *DNMT1* belongs to the drug-targeted down-regulated DEG.

exhibit down-regulated expressions (underlined words in Table 5), and two (12%) have unknown expression patterns. Based on the literature reviewing, it was found that most of the miRNAs (88%) are expressed in the CVD-associated diseases or tissues, and suggests these predicted results are reliable herein.

In the pathogenesis of AT, the HASMCs, oxLDL induced the significant expression of miR-29b, which mediated *MMP-2/MMP-9* and *DNMT3b* up-regulation and down-regulation, respectively. MiR-29b expression was reduced after silencing by antagomiR-29b.⁵⁷ This result suggests miRNA-based therapeutic means of treating the VSMC-associated diseases by inhibiting miRNA expression. One study suggested that anti-miR-27b and anti-miR-148a can down-regulate the expression of miR-27b and miR-148a, which can feasibly be used to treat CVDs.⁷⁵ The above studies provide a new perspective on the use of anti-miRs as anti-disease drugs, which play an important role in research into the miRNA-based treatment of CVDs.

4. Discussion

Several studies have suggested that the VSMC-associated diseases and cancer have similar developmental processes.^{8–10} Both diseases share several molecular pathways.

In this work, processing sets of DEGs with and without ClusterONE to query cMap, the former method yielded a higher hit ratio of drugs with measured IC₅₀ activities. Some of the identified drugs are undergoing clinical trials. Also, one miRNA-regulated DEG, *DNMT1*, whose miRNAs are potential targets for developing anti-miR therapy, was identified. There are 37 drugs for potential repurposing and 17 putative anti-miRs were identified for treating the oxLDL-induced VSMC-associated diseases. Some of the findings are supported by *in vitro* and clinical trial data that demonstrate the feasibility of the proposed approach.

Although important results were obtained, this work has some limitations. First, to improve the hit ratio of identification of drugs with measured IC₅₀ activities, ClusterONE was used. Since both the up- and down-regulated clusters that were

identified by ClusterONE are small, no drug-targeted DEG was identified. Secondly, the ClusterONE algorithm was not benchmarked against other clustering algorithms. Thirdly, although the identifications of the drugs were well supported by *in vitro* and clinical trial data, the cMap drugs, which were tested against cancer cells, may be toxic to VSMCs. Finally, antimiR-based therapeutics have not yet been tested in a clinical setting.

5. Conclusions

To examine how VSMCs react to oxLDL stimulation, a systematic study was carried out. First, *in silico* analysis was performed to examine how VSMCs respond to oxLDL perturbation and time-course microarray data were used to identify DEGs. Next, the identified DEGs were used to perform enrichment analysis to identify the most relevant BP and pathway terms. The cMap resource was used to identify potential drugs against the VSMC-associated diseases. Then, the DrugBank and NCBI PubChem Compound resources were utilized to identify drug-targeted DEGs for probing the course of gene therapy. Finally, the miRTarBase and TarBase resources were used to investigate the miRNA-target interactions, enabling miRNA-regulated DEGs to be identified.

In conclusion, the potential of existing drugs and antimiRs for inhibition of oxLDL-induced VSMC-associated diseases was assessed, potentially supporting the repurposing of drugs and the development of alternative therapeutic strategies. Furthermore, *in vitro* and *in vivo* studies are required to explore the potential for using either existing drugs or antimiRs against the VSMC-associated diseases.

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