Computational Identification of Candidate Genes Involved in the Inflammation Pathway of AGE-RAGE Signaling in Lung Cancer Invasiveness

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***Abstract*—Due to its high mortality rate, cancer has been at the forefront of biomedical research. Specifically, lung cancer has been an ongoing public health problem as it accounts for the highest number of cancer deaths worldwide. Phenotypic expressions such as the invasiveness of lung cancer can vary among individuals. Studies have shown a strong correlation between lung cancer invasiveness and inflammation pathways, making inflammation an ideal biomarker for lung cancer diagnostics and a target for therapeutics. The AGE-RAGE signaling pathway, which causes inflammation by genes with differential expression, is one such pathway for inflammation. Typical causing factors include but are not limited to reactive oxygen species, miRNA targets, mutational signatures, and other pathological functions. Even though the AGE-RAGE inflammation pathway is influential in cancer, it has not yet been developed in therapeutics for cancer invasiveness and should be further studied. In this study, we evaluated the complexities of the AGE-RAGE pathway expression in cancer. Through a detailed investigation using online genomics databases, bioinformatics data analysis, and literature review, the role of inflammation is systematically explored. We also examined the implications of the upregulation of AGE-RAGE on other pathological functions and diseases.**

***Keywords*—AGE-RAGE, cancer, inflammation, miRNA**

I. Introduction

Lung cancer, a highly invasive and pervasive cancer, is the leading cause of cancer-related mortality in the United States and worldwide, accounting for 18% of all cancer deaths. Among men, lung cancer is the leading cause of cancer mortality. Among women, it ranks third, attributing the primary cause, in both men and women, to be smoking [1]. Lung cancer is categorized as Small-cell Lung Cancer (SCLC) or Non-small-cell Cancer (NSCLC). NSCLC represents about 80% to 85% of all lung cancers and includes the subtypes of large cell carcinoma, squamous cell carcinoma, and adenocarcinoma. SCLC is less common, and tends to metastasize faster than NSCLC, and has a higher mortality rate [2]. Cancer invasiveness is a significant avenue in lung cancer studies as certain tumors can metastasize faster, leading to higher mortality. Invasiveness refers to cancer's ability to spread beyond the tissue it had developed into healthy tissue and thus infiltrating the body.

Clinical and epidemiological studies indicate a strong association between inflammation and lung cancer [3]. Inflammation is involved in the stages of tumorigenesis, especially in malignant tumors. When the immune system senses a neoplasm, it sends out inflammatory cells and cytokines that increase the cancer cell's resistance to apoptosis and increases proliferation [3]. Malignant neoplasms also tend to arise in microenvironments of chronic inflammation, causing proliferation, angiogenesis, immune escape, and carcinogenesis. As such, in past studies, researchers have recognized chronic inflammation as a hallmark of cancer growth [4]. Inflammatory components of cancer include chemokines, cytokines, and cytotoxic mediators, such as Reactive Oxygen Species (ROS) [5]. ROS is a free radical and unstable molecule that contains oxygen and can impair physiological function. A buildup of ROS can cause damage to macromolecules, including DNA, RNA, proteins, and lipids, which can further induce cancer growth [6]. Many factors can trigger the creation of excess ROS, such as Advanced Glycation End products (AGEs) [7].

AGEs are modified proteins or lipids that become glycated after exposure to sugars, forming glycosylated molecules. AGEs can cause cascades leading to cellular dysfunctions and modulation of gene expression via signaling from the receptor for Advanced Glycation End products (RAGE) [8]. RAGE, a membrane-bound protein, is a pro-inflammatory pattern recognition receptor that triggers intracellular signaling leading to the expression of inflammation molecules [9]. Accordingly, the AGE ligand binds to the RAGE receptor, initiating a signal transduction cascade called the AGE-RAGE signaling pathway. The pathway can cause a positive feedback loop by synthesizing more RAGE and producing more inflammation. AGE-RAGE signaling also creates ROS as a byproduct. Furthermore, inflammation in RAGE signaling is integral in other lung diseases, such as

Manuscript received January 19, 2023; revised February 22, 2023; accepted July 18, 2023.

pulmonary fibrosis, chronic obstructive pulmonary disease, asthma, and cystic fibrosis [10]. Gene expression of the AGE-RAGE pathway should be further studied to target cancer therapeutics.

Upstream of gene expression are regulatory microRNAs (miRNA) that control the production of proteins. MiRNA is small segments of non-coding RNA that regulate gene expression, causing differential gene expression. Regulatory functions are completed through the RNA-Induced Silencing Complex (RISC) that targets mRNA. In observations, researchers have indicated that the expression of miRNA is altered in tumors, implying that miRNA may be a factor in cancer development specifically in cell proliferation, differentiation, or apoptosis [11]. MiRNA targets of the AGE-RAGE pathway may prove to be significant targets to minimize invasiveness.

Although the AGE-RAGE signaling pathway shows promising connections to lung cancer, the pathway has not yet been targeted in lung cancer patients of pediatric and adult populations; hence, further gene expression and miRNA research is required on this pathway to develop new therapeutic agents for lung cancer patients [10].

The goal of this study is to examine the patterns of AGE-RAGE pathway expression in lung cancer. To do so, we conducted differential gene expression and microRNA analysis to explore the pathway's impact on tumorigenesis.

II. Methods

The overall method workflow is shown in Fig. 1.

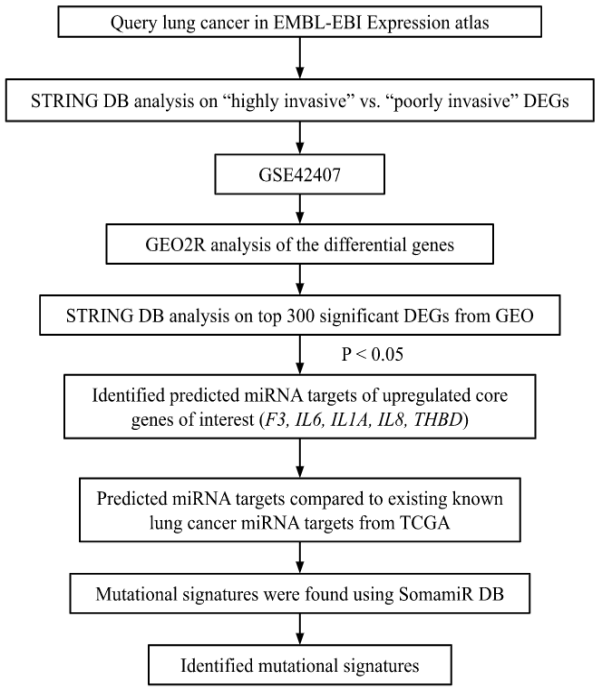


Figure 1. Workflow diagram of bioinformatics analysis of publicly available datasets from GEO and TCGA databases used in this study.

A. EMBL-EBI Expression Atlas Data

EMBL-EBI Expression Atlas [12] was used to query lung cancer cell expression in homo sapiens to find Differentially Expressed Genes (DEGs). The DEGs were filtered by their comparison variables: lung cancer vs. normal lung cells, highly invasive vs. poorly invasive, and malignant vs. non-malignant tumors. The DEGs from each comparison group were queried through STRING [13] to search protein-protein interactions and enrichment pathways. The comparison of highly invasive to poorly invasive lung cancer cells became the focus of the study because protein-protein interactions between the CL1-0 and CL1-5 cell lines displayed the most interactions through the nodes in STRING-DB (Fig. 1).

B. Microarray Dataset GSE42407

Poorly invasive vs. highly invasive lung cancer data from EMBL-EBI data originated from National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) [14, 15] database GSE42407. GSE42407 consisted of 6 samples: 3 poorly invasive CL1-0 cell lines (GSM1039261, GSM1039262, GSM1039263), and 3 highly invasive CL1-5 cell lines (GSM1039264, GSM1039265, GSM1039266). Data was gathered through microarray expression profiling with platform GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The CL1-0 and CL1-5 cell lines were obtained from a lung cancer cell model of invasive transformation of increasingly invasive cell populations from a parental cell line, CL1, of NSCLC. Clones were named CL1-1, CL1-2, CL1-3, CL1-4, and CL1-5. Expression profiling by array was used to compare expression profiles of CL1-0, being poorly invasive, and CL1-5, being highly invasive, to identify invasion of associated gene signatures [16].

C. Data Processing with R and GEO2R

The series matrix file was downloaded and run in RStudio (v4.1.3) [17] using the library pheatmap package to generate a heatmap of the DEGs [18]. GEO2R was used to identify DEGs and generate visualizations. The DEGs were downloaded to a top table file and filtered out all non-protein-encoding genes by their GenBank accession number. The top 300 statistically significant genes ($p < 0.05$) were queried through STRING to find enrichment pathways. DEG enrichment analysis was performed on both datasets from EMBL-EBI Expression Atlas and GEO.

D. Gene Set Enrichment Pathway Analysis

The gene-to-pathway interactions were analyzed through KEGG Pathways. KEGG is a database used to understand high-level functions of biological interactions through network diagrams [19–21]. Further research was performed on the indicated protein-protein interactions and pathways through a literature review and genomic databases. The Genotype-Tissue Expression Project (GTEx portal) was used to identify the relationships between gene expression of different tissues to solidify the impact of differential gene expression on lung tissue. Kaplan Meier plots were generated to visualize the survival trends of the genes to support the oncogenic nature of the candidate genes.

E. MiRNA and Mutation Analysis

Further analysis were conducted to identify miRNA interaction clusters among the DEGs and mutations that would disrupt the regulatory process of miRNA. Using the TargetScan database [22], miRNA data of the predicted (conserved) targets of miRNA families were downloaded to compare to the miRNA of the core 5 upregulated DEGs. The upregulated genes of interest (*F3*, *IL6*, *IL1A*, *IL8*, *THBD*) were queried and the respective target miRNA for each gene was identified. These miRNA targets were compared with existing Lung Adenocarcinoma (LUAD) cluster miRNA interaction pairs [23]. Congruent miRNA targets were identified and queried through SomamiR DB [24] to find mutations specific to the gene.

III. Results

A. Protein-Protein Interaction and Pathway Analysis for Genes in the AGE-RAGE Pathway

STRING analysis of DEGs from Expression Atlas revealed significant molecular functions and metabolic pathways, such as chemokine and cytokine activity, viral protein interaction with cytokine receptors, and the AGE-RAGE signaling pathway in diabetic complications. This study identified 6 core genes (AGTR1, F3, IL6, IL1A, IL8, and THBD) strongly linked to the AGE-RAGE signaling pathway (Table I).

TABLE I. DEGs in the AGE-RAGE Pathway from EBI Expression Atlas

Gene Symbol	Ensembl Gene ID	Fold Change	Adjusted p-value
AGTR1	ENSG00000144891	−5.7	1.48E-09
F3	ENSG00000117525	6.1	1.10E-09
IL6	ENSG00000136244	6.9	3.92E-09
IL1A	ENSG00000115008	7	5.82E-10
IL8	ENSG00000169429	7.9	4.94E-10
THBD	ENSG00000178726	8.1	5.82E-10

The enriched pathways were determined through analysis methods using Expression Atlas, STRING, and KEGG pathway databases. From the query of highly vs. poorly invasive lung cancer in STRING, the AGE-RAGE signaling pathway in diabetic complications (strength = 1.11, FDR = 0.00100, count in network = 6 of 98) showed the most promising results from its calculated statistical significance. The genes of interest were contained in the same inflammation pathway in AGE-RAGE signaling.

B. Gene Expression Difference for Studied GEO Samples

Further analysis of GSE42407 shows a strong distinction between upregulated and downregulated genes as seen from the heatmap generated in RStudio (Fig. 2).

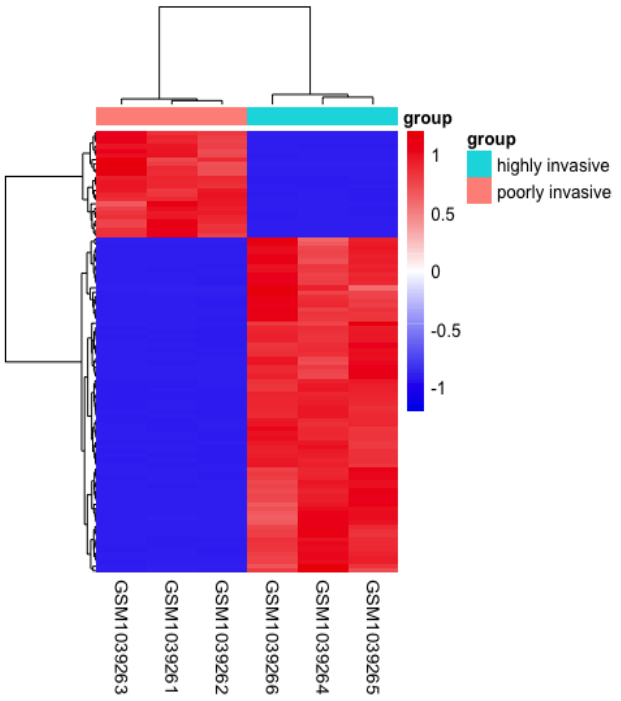


Figure 2. Heatmap of the top 300 differentially expressed genes in the 6 cell line samples.

The UMAP generated from GEO2R analysis showed clear clustering and distinction between the experimental groups (Fig. 3). Secondary analysis utilizing GEO2R with GSE42407 showed similar results in regard to the genes of interest. GEO2R analysis identified 54,675 DEGs. Through a query of the top 300 significant genes, the AGE-RAGE pathway was also identified as significant (strength = 0.92, FDR = 5.55e-06, count in network = 12 of 98).

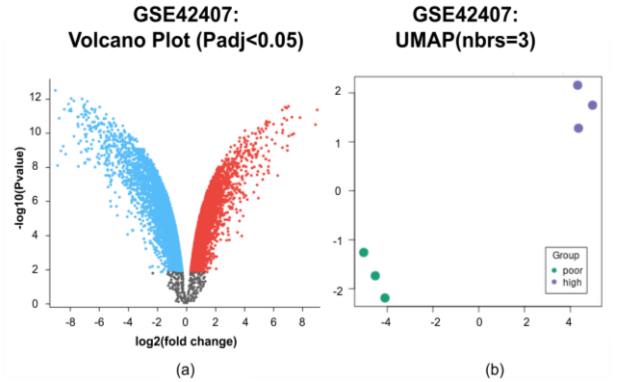


Figure 3. Volcano Plot (a) and UMAP (b) of the samples in GSE42407 show distinctive clustering, separation, and no outliers.

C. Functional Analysis for GEO Datasets

Biological processes identified through STRING analysis are similar to the Expression Atlas analysis result, specifically, cytokine-mediated signaling pathways and positive regulation of angiogenesis were reported as enriched pathological functions. The genes AGTR1, IL8, IL6, THBD, and F3 overlap between the two analyses. New candidate genes include ICAM1, AP-1, IL1B, SMAD3, PAI-1, STAT1, and COL (Table II). Many of the

newly identified genes are also involved in the inflammation pathway (Fig. 4).

TABLE II. DEGS IN THE AGE-RAGE PATHWAY FROM GEO2R ANALYSIS

Gene Symbol	Ensembl Gene ID	Fold Change	Adjusted p-value
AGTR1	ENSG00000144891	−4.09	1.30E-06
ICAM1	ENSG00000090339	2.62	8.88E-07
THBD	ENSG00000178726	6.29	2.59E-08
AP-1	ENSG00000177606	2.72	1.55E-06
IL8	ENSG00000169429	7.94	1.04E-08
IL6	ENSG00000136244	6.88	5.02E-08
IL1B	ENSG00000125538	5.17	2.28E-07
SMAD3	ENSG00000166949	3.41	6.07E-07
PAI-1	ENSG00000106366	4.82	7.89E-08
F3	ENSG00000117525	6.09	1.62E-08
COL	ENSG00000187498	2.34	1.70E-06
STAT1	ENSG00000115415	2.93	3.54E-07

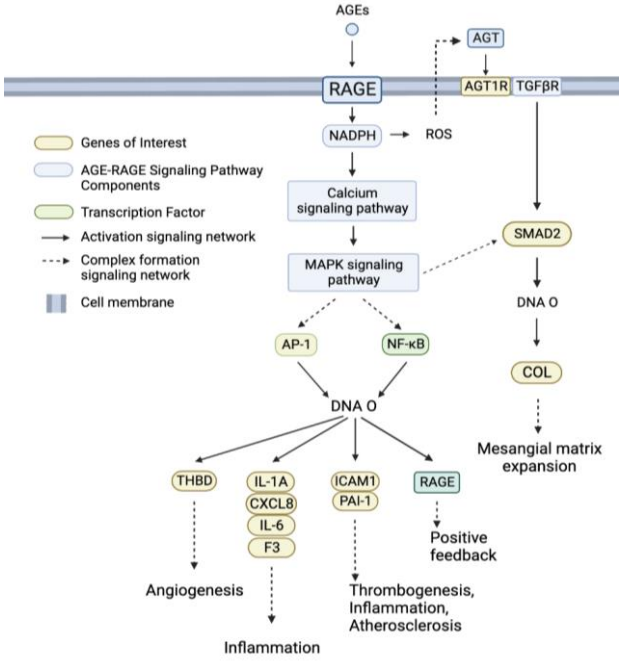
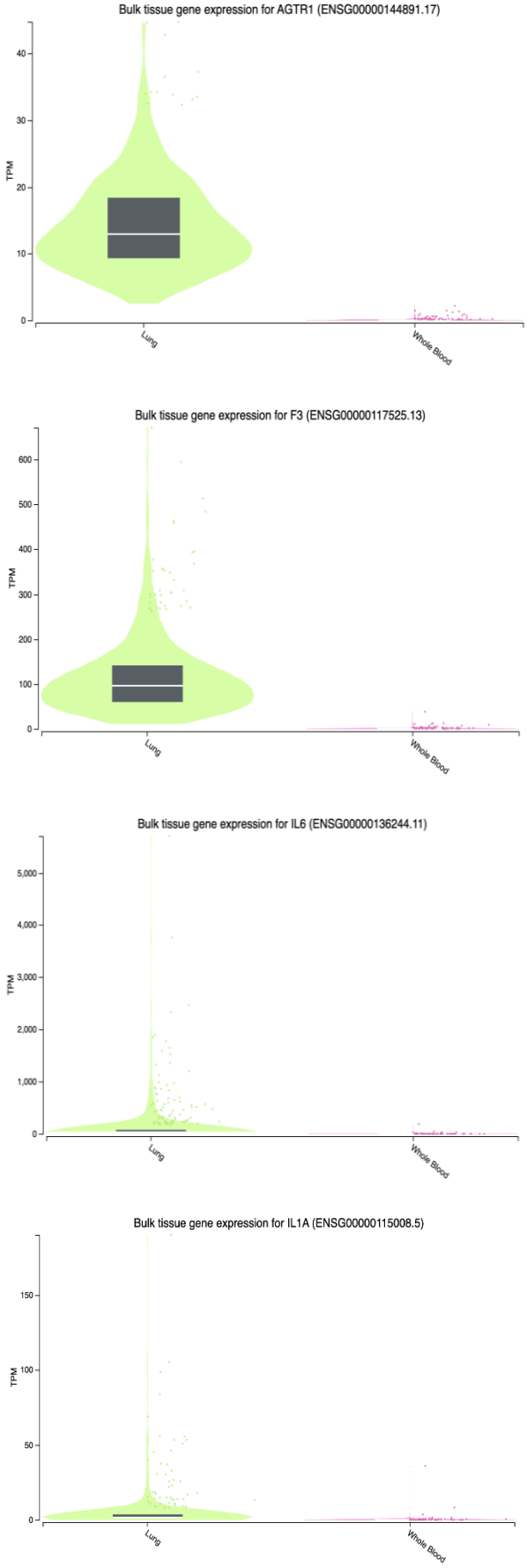


Figure 4. AGE-RAGE signaling pathway (template figure was taken from KEGG database).

The upregulated genes in the AGE-RAGE pathway all contributed to an elevated inflammation pathway in patients with highly invasive lung cancer. The genes showed higher expression in lung tissue when compared to whole blood tissue, resulting in statistical significance for the higher expression (Fig. 5).



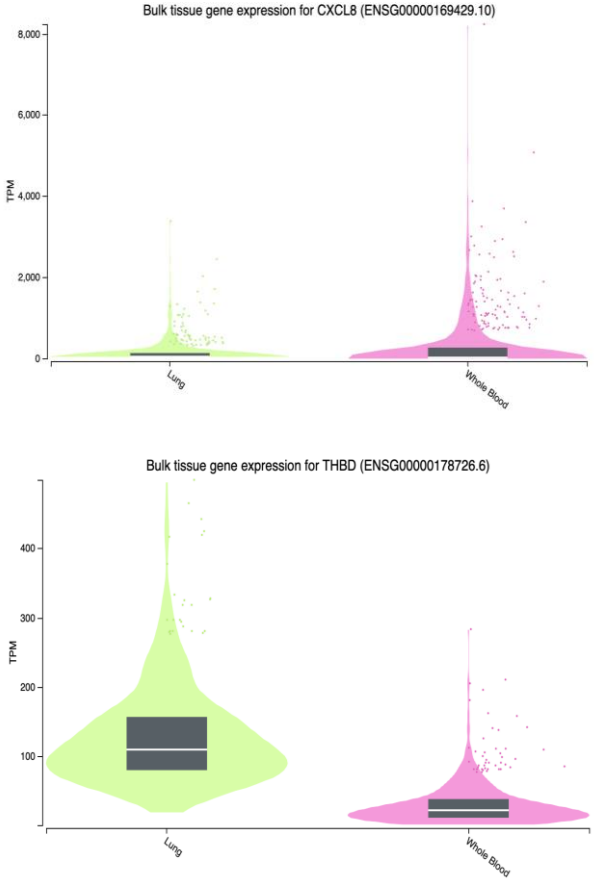


Figure 5. Bulk tissue gene expression data of DEGs from GTEx Portal based on a comparison between lung tissue (green) and whole blood (pink) transcript per million cells.

The gene expression was compared in lung tissue and whole blood. Whole blood was used as a comparison because of its accessibility in sample collection and relative convenience in performing tests. The visualizations depict a significantly higher Transcript per Million cells (TPM) value in lung tissue for the majority of the genes. Significantly greater expression in the lung tissue supports the approach of finding enrichment pathways using bioinformatics analysis of DEGs is valuable. Thus, the expression data confirm the role of the inflammation pathway in AGE-RAGE signaling being significant as these genes are expressed in abundant amounts in the lung.

D. Survival Analysis

DEGs were also determined to be significant oncogenes according to Kaplan-Meier plots [25]. Genes that showed a clear distinction between low and high survival include AGTR1, IL1A, IL6, and IL8. Low expression of AGTR1 (p = 1.2E-10) was seen to decrease the survival rate over a period of time. High expression of IL1A (p = 9.4E-0.8), IL6 (p = 2.0E-0.5), and IL8 (p = 1.4E-0.4) was reported to have a decreased survival trend in lung cancer over time (Fig. 6).

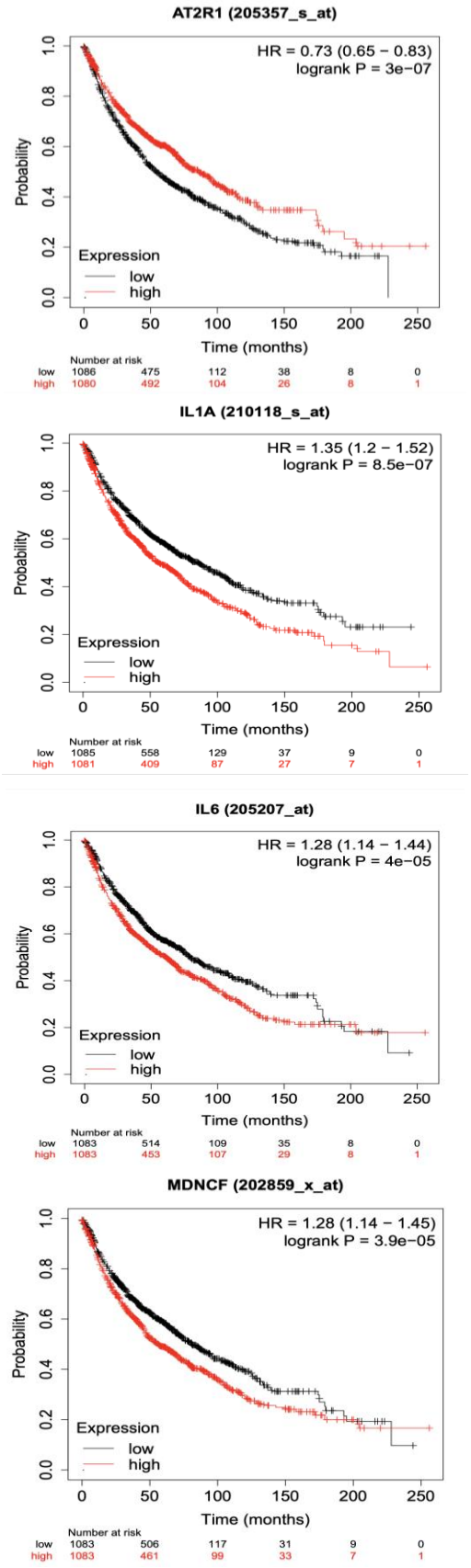


Figure 6. Kaplan-Meier survival plots for *AGTR1*, *IL1A*, *IL6*, *IL8 (MDNCF).*

E. Mutation Pattern for Targeting miRNA Based on TCGA LUAD Cluster Results

Comparison between our miRNA analysis and a previously published study [23] cluster 14 result (p-value = 0.009199, q-value = 0.1931) found miRNA target of *F3*. Interestingly, its targeting miRNA- *hsa-mir-148a* predicted by TargetScan [22] was also found both in the LUAD cluster data. Additionally, the GTEx gene expression results for *mir-148a* were expressed higher in the lung compared to other tissues (Fig. 7). SomamiR [24] results also report a mutation for *mir-148* gene, occurring at chr7:g.25949959C>T and chr7:g.25949963G>T.

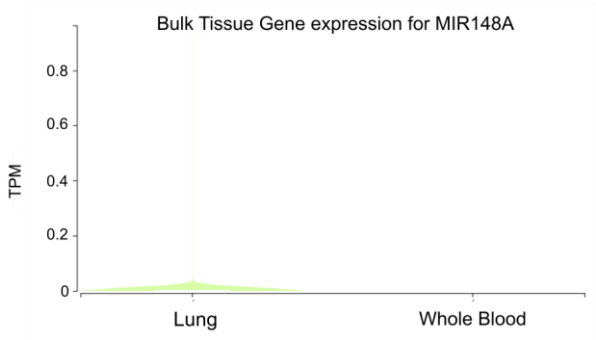


Figure 7. Bulk tissue gene expression data of *mir148a* from GTEx Portal based on a comparison between lung tissue (green) and whole blood in transcript per million cells.

Additionally, miRNA target hsa-mir-181 was also identified from LUAD cluster data in Cluster 10 (p-value = 1.00E-0.5, q-value = 3.00E-05) from the same study [23]. The two identified were hsa-mir-181c and hsa-mir-181d. Using SomamiR DB, we identified the mutation in IL8, chr4:g.73743195A>G, that could alter predicted mRNA-miRNA target sites. Hsa-mir-181 has become an interest for its regulation of key biological processes, including embryonic development, cell proliferation, apoptosis, autophagy, mitochondrial function, and immune response. Although cluster analysis identified mir-181c/d as targets, conclusions regarding mir-181a/b can be broadened to the mir-181 family as mir-181a and mir-181c only differ in one deletion (Table III). Mir-181b and mir-181d differ by one base. Mir-181 members are evolutionarily conserved where mir-181a/b paralogs are conserved across all vertebrate species and paralogs mir-181c/d likely evolved more recently due to independent evolution [26].

TABLE III. SEQUENCE ALIGNMENT OF MIR-181 FAMILY

MicroRNA	Sequence
hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-181c-5p	AACAUUCAAC – CUGUCGGUGAGU
hsa-miR-181b-5p	AACAUUCAUUGCUGUCGGUGGGU
hsa-miR-181d-5p	AACAUUCAUUGUUGUCGGUGGGU

A recent study done on 2,653 lung cancer patients identified mir-181a to have significant expression levels correlated with survival through downregulation. Mir-181a inhibits lung cancer proliferation and migration by targeting VCAM1. As shown in the previous GEO2R analysis, ICAM1 was found to be a downregulated DEG. The authors also demonstrated that the pro-inflammatory cytokine Interleukin (IL)-17 and the NF-κB pathway control the interaction between miR-181a and VCAM1 [26]. VCAM1 and ICAM1 are similar in structure and function [27]. Additional studies show that the mir-181 family affects spermatogenesis, specifically through the target of S6K1. Mir-181a/b was upregulated in mouse spermatogenesis and may cause infertility in male patients with lung cancer through downregulation. Mir181c is seen to have the greatest expression in the male testis (Fig. 8). Kaplan Meier analysis showed patients with high S6K1 levels showed worse survival than those with low S6K1 levels, and thus, overexpression in lung cancer patients has the potential to cause cancer metastasis [28].

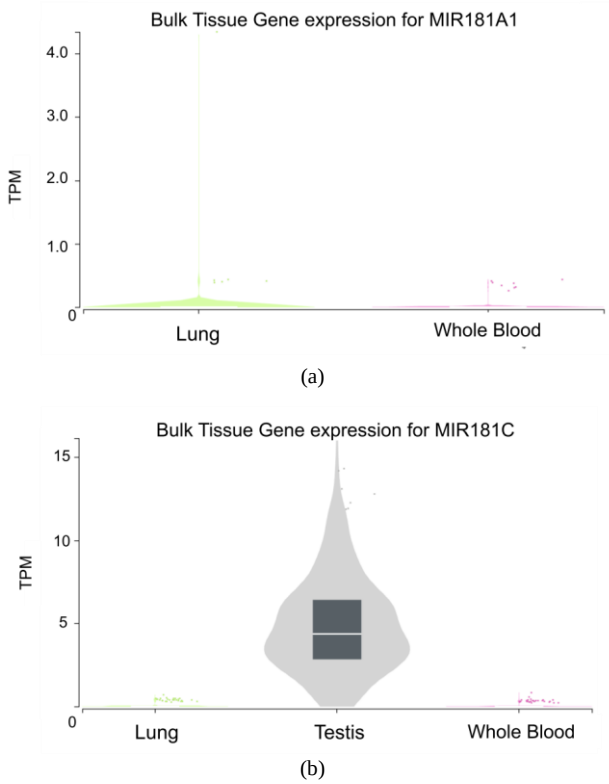


Figure 8. Bulk tissue gene expression data of *mir181* family, *mir181a* (a) and *mir181c* (b) from GTEx Portal based on a comparison between lung tissue (green) and testis tissue (gray) and whole blood in transcript per million cells.

IV. DISCUSSION

Inflammation of the AGE-RAGE pathway is a pivotal component of highly invasive lung cancer. As AGE binds to its receptor, RAGE, a signal transduction cascade is induced, creating a byproduct of ROS and aldosterone [10, 29]. Aldosterone also induces the formation of ROS. The downregulation of AGTR1 inhibits the cell's secretion properties and prohibits the efficient ejection of aldosterone, resulting in a buildup that further stimulates the formation of ROS, triggering pro-inflammatory transcription factors [29]. Free radicals bind and oxidize

DNA. DNA damage then accumulates, causing carcinogenesis via genomic instability. Additionally, DNA repair responses to the damaged DNA can induce inflammation, creating an inflammatory microenvironment. Thus, inflammation plays an integral role in cancer development and is an important biomarker of precancerous symptoms. ROS causes inflammation and dysregulates the membrane regulator, causing inflammation by inhibiting access to movement across a cell membrane [30].

NF-κB protein is a member of a family of transcription factors that cause inflammation [9]. When ROS is generated through the AGE-RAGE signaling, it activates NF-κB and other inflammatory mechanisms. NF-κB induces the expression of pro-inflammatory genes, such as IL6 and IL8, further exacerbating the inflammatory response. NF-κB can also bind to the gene that encodes for RAGE to promote AGE-RAGE expression and create a positive feedback loop for chronic infection [10].

AP-1 is another transcription factor that orchestrates gene expression of inflammation, oncogenesis, and apoptosis. *AP-1* is induced by an extracellular signaling-regulated kinase (ERK), a subgroup of the MAPKs [31]. MAPK signaling pathway is involved in similar functions, including apoptosis, proliferation, and metastasis [32]. Additionally, in vivo studies involving ROS, NF-κB, and *AP-1* in mice, researchers have reported that the generation of ROS stimulates transcription by activating *AP-1* and NF-κB. Both are activated through the MAPK pathway and ultimately lead to the translation of inflammatory proteins [31].

Parallel pathological functions from the AGE-RAGE pathway can be observed in lung cancer patients, including thrombogenesis, angiogenesis, and atherosclerosis. Thrombosis can be caused by and trigger inflammation [33]. A case study found an increased incidence of thromboembolic events in adenocarcinoma patients [34]. AGE-RAGE also promotes angiogenesis which allows for the formation of new inflammatory vessels and cancer cell vascularization [35]. Because all these pathological functions stem from the AGE-RAGE pathway, targeting the genetic background of these mechanisms may prove beneficial for minimizing inflammation in lung cancer and mitigating cancer invasiveness.

Although the upregulation of the inflammation pathway of the AGE-RAGE pathway in lung cancer should imply that the entire AGE-RAGE pathway is upregulated in highly invasive NSCLC, other studies show that RAGE takes on a contrasting role in lung malignancies. Paradoxically, some studies have shown overexpression of RAGE in lung cancer cells reduces proliferation and tumorigenesis [10]. Accordingly, although the AGE-RAGE pathway can mediate the inflammation pathway through the production of ROS, RAGE signaling can also mitigate tumorigenesis under certain circumstances [10].

IL6, *IL1A*, *IL1B*, and *IL8* are all upregulated and part of the interleukin cytokine family. These genes are involved in the inflammatory process, immune responses, hematopoiesis, cell proliferation, differentiation, and apoptosis [36]. Because of its interplay in the inflammatory pathway, *IL-1* is reported to be associated with aggressive cancer phenotype and has become a promising therapeutic target. Studies show that a positive correlation between *IL-1* production and metastasizing melanoma suggested that *IL-1* acts as a growth factor that enhances the synthesis of other cytokines or chemokines [37]. *IL1A* can induce endothelial cells to express *ICAM1*, which promotes the trans-endothelial migration of inflammatory cells [37]. *ICAM1* is an intercellular adhesion molecule that is significant in atherosclerosis. Previous studies have found *ICAM1* to be exclusively expressed gene subtypes with known association with inflammation and aggressive phenotypes [38]. Moreover, it was proven that breast cancer cell lines expressed *ICAM1* upon stimulation of proinflammatory cytokines [38]. Similar mechanisms may be applied to lung cancer.

F3 is a Coagulation Factor III that encodes for *TF* (Tissue Factor). *F3* is a cell-surface glycoprotein that is expressed as a cell-surface glycoprotein and as microparticles. *F3* and its coagulation factor VII activate the coagulation cascade [39]. *THBD* is tied to vascular regulation, allowing cancer tumors to create their vascular networks to obtain nutrition to grow [36, 40]. In a cohort with *F3*, *F3* initiates the coagulation cascade while thrombin can act as a positive feedback loop that magnifies coagulation and inflammation [36]

Upregulation of *F3* in lung cancer is also present in COVID-19 in a study showing a 5.2-fold increase in *F3* tissue factor gene causes extraneous coagulation through single-cell RNA sequencing from severe COVID-19 patients. Additionally, an additional 4.4-fold increase in THBD expression levels. *F3* and *THBD* transcript levels likely indicate that these genes are contributors to coagulation and thrombosis related to COVID-19, and upregulation of these genes is a significant indicator of related mortality in both COVID-19 as a coagulation factor and in lung cancer as an oncogene [41].

Moreover, based on the miRNA analysis, hypotheses can be made that highly invasive lung cancer may cause infertility in male patients. Because males are observed to smoke more than females with an estimated 170,000 smoking males and 126,000 smoking females in 2020 [42], *mir181c* may have evolved only in males because of the increased smoking rates and oxidative stress.

In this study, we have identified several novel miRNA targets involved in the AGE-RAGE pathway, which could exacerbate inflammation. The mutations that we found in miRNAs are important but yet to be further evaluated.

V. Conclusion

Through a bioinformatics approach analysis, the AGE-RAGE signaling pathway was found to be a significant component of the invasiveness of lung cancer. We explored the downstream pathological functions of AGE-RAGE signaling, miRNA targets, and mutational signatures. Further analysis can be conducted on the impact of the AGE-RAGE pathway in lung cancer on COVID-19 and male infertility. The AGE-RAGE inflammatory pathway is significant in lung cancer invasiveness but has not yet been targeted for cancer

treatment. Further evaluations based on the results of this study are necessary to solidify the potential for the AGE-RAGE pathway to be used as a biomarker in the diagnosis of lung cancer and future cancer interventions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GW conducted the research, analyzed the data, wrote the paper; YB provided guidance, reviewed the paper; all authors had approved the final version.

FUNDING

The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from: GTEx Portal on 06/14/2022.

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