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Integrated microRNA, gene expression and transcription factors signature in papillary thyroid cancer with lymph node metastasis

Nurul-Syakima Ab Mutalib, Sri Noraima Othman, Azliana Mohamad Yusof, Shahrun Niza Abdullah Suhaimi, Rohaizak Muhammad, Rahman Jamal

Background: Papillary thyroid carcinoma (PTC) is the commonest thyroid malignancy originating from the follicle cells in the thyroid. Despite a good overall prognosis, certain high-risk cases as in those with lymph node metastasis (LNM) have progressive disease and poorer prognosis. MicroRNAs are a class of non-protein-coding, 19-24 nucleotides single-stranded RNAs which regulate gene expression and these molecules have been shown to play a role in LNM. The integrated analysis of miRNAs and gene expression profiles together with transcription factors (TFs) has been shown to improve the identification of functional miRNA-target gene-TF relationships, providing a more complete view of molecular events underlying metastasis process. **Objectives**: We reanalyzed The Cancer Genome Atlas (TCGA) datasets on PTC to identify differentially expressed miRNAs/genes in PTC patients with LNM-positive (LNM-P) versus lymph node negative (LNN) PTC patients and to investigate the miRNA-gene-TF regulatory circuit that regulate LNM in PTC. **Results**: PTC patients with LNM (PTC LNM-P) has significantly shorter disease-free survival rate compared to PTC patients without LNM (PTC LNN) (Log-rank Mantel Cox test, p = 0.0049). We identified 181 significantly differentially expressed miRNAs in PTC LNM-P versus PTC LNN; 110 were upregulated and 71 were downregulated. The five topmost deregulated miRNAs were hsa-miR-146b, hsa-miR-375, hsa-miR-31, hsa-miR-7-2 and hsa-miR-204. In addition, 395 miRNAs were differentially expressed between PTC LNM-P and normal thyroid while 400 miRNAs were differentially expressed between PTC LNN and normal thyroid. We found 4 significant enrichment pathways potentially involved in metastasis to the lymph nodes namely oxidative phosphorylation (OxPhos), cell adhesion molecules (CAMs), leukocyte transendothelial migration and cytokine-cytokine receptor interaction. OxPhos was the most significantly perturbed pathway (p = 4.70E-06) involving downregulation of 90 OxPhos-related genes. Significant interaction of hsa-miR-301b with HLF, HIF and REL/NFkB transcription factors were identified exclusively in PTC LNM-P versus PTC LNN. **Conclusion**: We found evidence of five miRNAs differentially expressed in PTC LNM-P. Alteration in OxPhos pathway could be the central event in metastasis to the

lymph node in PTC. We postulate that hsa-miR-301b might be involved in regulating LNM in PTC via interactions with HLF, HIF and REL/NFkB. To the best of our knowledge, the roles of these TFs have been studied in PTC but the precise role of this miRNA with these TFs in LNM in PTC has not been investigated.

Integrated microRNA, gene expression and transcription factors signature in papillary thyroid cancer with lymph node metastasis

Nurul-Syakima Ab Mutalib[1*#], Sri Noraima Othman[1#], Azliana Mohd Yusof[1], Shahrun Niza Abdullah Suhaimi[2], Rohaizak Muhammad[2], Rahman Jamal[1]

[1]UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, 56000 Cheras, Kuala Lumpur, Malaysia

[2]Department of Surgery, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abd Aziz, 50300, Kuala Lumpur, Malaysia

Corresponding Author:
Nurul-Syakima Ab Mutalib
UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, 56000 Cheras, Kuala Lumpur, Malaysia

Email address: syakima@ppukm.ukm.edu.my

Abstract

Background. Papillary thyroid carcinoma (PTC) is the commonest thyroid malignancy originating from the follicle cells in the thyroid. Despite a good overall prognosis, certain high-risk cases as in those with lymph node metastasis (LNM) have progressive disease and poorer prognosis. MicroRNAs are a class of non-protein-coding, 19-24 nucleotides single-stranded RNAs which regulate gene expression and these molecules have been shown to play a role in

LNM. The integrated analysis of miRNAs and gene expression profiles together with transcription factors (TFs) has been shown to improve the identification of functional miRNA-target gene-TF relationships, providing a more complete view of molecular events underlying metastasis process.

Methods. We reanalyzed The Cancer Genome Atlas (TCGA) datasets on PTC to identify differentially expressed miRNAs/genes in PTC patients with LNM-positive (LNM-P) versus lymph node negative (LNN) PTC patients and to investigate the miRNA-gene-TF regulatory circuit that regulate LNM in PTC.

Results. PTC patients with LNM (PTC LNM-P) has significantly shorter disease-free survival rate compared to PTC patients without LNM (PTC LNN) (Log-rank Mantel Cox test, p = 0.0049). We identified 181 significantly differentially expressed miRNAs in PTC LNM-P versus PTC LNN; 110 were upregulated and 71 were downregulated. The five topmost deregulated miRNAs were hsa-miR-146b, hsa-miR-375, hsa-miR-31, hsa-miR-7-2 and hsa-miR-204. In addition, 395 miRNAs were differentially expressed between PTC LNM-P and normal thyroid while 400 miRNAs were differentially expressed between PTC LNN and normal thyroid. We found 4 significant enrichment pathways potentially involved in metastasis to the lymph nodes namely oxidative phosphorylation (OxPhos), cell adhesion molecules (CAMs), leukocyte transendothelial migration and cytokine-cytokine receptor interaction. OxPhos was the most significantly perturbed pathway (p = 4.70E-06) involving downregulation of 90 OxPhos-related genes. Significant interaction of hsa-miR-301b with HLF, HIF and REL/NFkB transcription factors were identified exclusively in PTC LNM-P versus PTC LNN.

Discussion. We found evidence of five miRNAs differentially expressed in PTC LNM-P. Alteration in OxPhos pathway could be the central event in metastasis to the lymph node in

PTC. We postulate that hsa-miR-301b might be involved in regulating LNM in PTC via interactions with HLF, HIF and REL/NFkB. To the best of our knowledge, the roles of these TFs have been studied in PTC but the precise role of this miRNA with these TFs in LNM in PTC has not been investigated.

Introduction

Papillary thyroid carcinoma (PTC) is the most common malignancy originating from the thyroid. Although the prognosis of PTC is generally good with a high 5-year survival rate, cases demonstrating certain clinicopathological parameters are progressive, have poorer prognosis and are considered as high-risk (Ito *et al*., 2009). Numerous classification systems for thyroid carcinoma have been established in order to classify high-risk cases such as AMES (Cady and Rosai, 1988), AGES (Hay *et al*., 1987), MACIS (Hay *et al*., 1993) as well as TNM (Sobin and Wittekind, 2002; AJCC 2010). The TNM classification is the most recent classification system and is based on size and extrathyroid extension (T), lymph node involvement (N), distant metastasis (M) and patient's age.

There are two regional lymph node compartments that are involved in PTC, namely the central and lateral compartments. The lymph node involvement (N) is further divided into a number of categories in the TNM classification (AJCC 2010); no regional node metastasis (N0), regional lymph node metastasis (N1), metastases to Level VI i.e. pretracheal, paratracheal, and prelaryngeal/Delphian lymph nodes (N1a), metastases to unilateral, bilateral, or contralateral cervical i.e. Levels I, II, III, IV, or V or retropharyngeal or superior mediastinal lymph nodes i.e. Level VII (N1b) (AJCC 7th edition, 2010). N1b is considered as the highest level of lymph node involvement. Despite the fact that PTC can metastasize to either compartment with a similar incidence (Ito *et al*., 2006), N1b cases are graded higher compared

to N1a cases in the TNM classification and are classified as stage IV regardless of other factors such as tumor size and extrathyroid extension if the patient is 45 years old or older (Voutilainen *et al*., 2001; Zaydfudim *et al*., 2008; Ito *et al*., 2009).

MicroRNAs (miRNAs), firstly identified in *Caenorhabditis elegans*, are a class of endogenous (non-protein-coding), 19-24 nucleotides single-stranded RNAs that derive from a stem-loop precursor to regulate gene expression by binding primarily to the 3'-UTR of specific 'target' messenger RNA (mRNAs). MiRNAs that bind with perfect or nearly perfect complementarity to protein-coding mRNA sequences induce the RNA-mediated interference (RNAi) pathway, resulting in the disruption of mRNA stability and/or translation (Bartel 2009). Dysregulation of miRNAs expression in human cancers have been demonstrated by many studies (Iorio and Croce, 2012). Through expression profiling studies, miRNAs were shown to be linked to tumor development, tumor progression, and response to treatment, signifying their potential use as biomarkers for diagnosis and prognosis (Iorio and Croce, 2012). MiRNAs have also been shown to play a role as biomarkers in predicting lymph node metastasis (LNM). There was a positive correlation between high hsa-miR-21 expression with tumor stage and LNM in patients with breast cancer (Yan *et al*., 2008), and the development of distant metastases in colorectal cancer patients (Slaby *et al*., 2007). Most recently, hsa-miR-1207-5p was suggested as a useful biomarker in the prediction of LNM in gastric cancer (Huang *et al*., 2015) and head and neck cancer (de Carvalho *et al*., 2015).

The current approach of miRNA target gene prediction via *in silico* analysis is built upon sequence similarity search and thermodynamic stability (Alexiou *et al*., 2009). Nevertheless, it is acknowledged that the results of *in silico* target prediction algorithms suffer from very low specificity (Alexiou *et al*., 2009). The combination of *in silico* target predictions with miRNA and gene expression profiles has been proven to improve the identification of functional miRNA-target gene relationships (Nunez-Iglesias *et al*., 2010; Ma *et al*., 2011). As miRNAs

act prevalently through degradation of the target genes, expression profiles of miRNA and target genes/transcripts are predicted to be inversely correlated (Bisognin *et al*., 2012). Another regulatory component, the transcription factors (TF), has also been shown to activate or repress miRNA expression level, further adding to the complexity of gene regulation. Efforts have been made to comprehend the mechanism of miRNAs in regulating target genes; however the study of miRNA regulation by TFs (TF–miRNA regulation) is rather limited (Wang *et al*., 2010a).

The Cancer Genome Atlas (TCGA) Research Network recently published a molecular characterization of 507 PTCs and 59 matched normal adjacent tissues with respect to genomic, transcriptomic and proteomic signatures together with DNA methylation profiles, clinical and pathological features (Cancer Genome Atlas Research Network, 2014). Data were collected through several studies across different institutions, thus creating a comprehensive dataset of PTC samples. Through unsupervised clustering methods, TCGA yielded six subtypes for miRNA expression and five for gene expression. However, miRNA and gene expression profiles between PTC with and without LNM were not comprehensively discussed. Here we reanalyzed these TCGA datasets on PTC with the aim of identifying differentially expressed miRNAs/genes in PTC patients with LNM-positive (LNM-P) as compared to lymph node negative (LNN) PTC patients and to investigate the miRNA-gene-TF regulatory circuit that governs LNM in PTC.

Materials and Methods

TCGA papillary thyroid cancer dataset

We used the TCGA-generated microRNA sequencing (miRNAseq) and mRNAseq data for 495 tumors and 59 adjacent normal samples. Metadata containing clinical information,

miRNAseq and mRNAseq of 507 PTC patients as well as *BRAF* V600E mutation status were obtained from the TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) [accessed from March 27, 2015 to May 25, 2015]. Information were available for 507 PTC patients. The list was then filtered for PTC with N0, N1, N1a, N1b as well as normal thyroid tissues as control. The clinical parameters are presented in Table 1.

Only samples with paired miRNAseq and mRNAseq data were selected, resulting in a total of 477 patients' dataset which includes 213 patients with PTC LNN (N0), 205 PTC LNM-P (53 patients with N1, 86 patients with N1a, 66 patients with N1b) and 59 normal thyroid tissues (Supplementary Table 1). The miRNA and gene expression datasets consisting of 1046 human miRNAs and 20531 genes, respectively, were used for subsequent analysis.

Survival analyses

Kaplan–Meier survival analysis was carried out on disease-free and overall survival duration of PTC patients for whom follow-up details were available. Curves were compared by univariate (log-rank) analysis. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad, San Diego, CA, USA). P values ≤ 0.05 were considered significant.

Bioinformatics analyses

The miRNASeq and RNASeq V2 level 3 data from TCGA were used exclusively. The normalised expression (reads per million or RPM) of all miRNAs was $\log_2$-transformed and used for fold change calculation. The RNAseq by Expectation-Maximization (RSEM) values (from files with the extension .rsem.genes.results) were used to quantify messenger RNA

(mRNA) expression levels. The RSEM algorithm is a statistical model that estimates RNA expression levels from RNA sequencing counts (Li and Dewey, 2011). We then performed the Students' unpaired t-test with a Benjamini Hochberg false discovery rate (FDR) multiple testing correction and $\log_2$ fold change calculation using Bioconductor version 3.1 (BiocInstaller 1.18.2) (Gentleman *et al.*, 2011) in R version 3.2.0 (R Development Core Team, 2008). Downregulated genes will have negative $\log_2$ values while upregulated genes will have positive $\log_2$ values. Statistical significance is denoted as $p \leq 0.05$. Heatmaps were created using GeneE from the Broad Institute (http://www.broadinstitute.org/cancer/software/GENE-E) while Venn diagrams were created using Venn online tool (http://bioinformatics.psb.ugent.be/webtools/Venn). All other figures were created or labelled using Adobe Photoshop.

Pathway enrichment analysis and integrated analysis of miRNA and gene expression

The functions and pathways of the differentially expressed genes were annotated and analysed using the annotation tools from the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al.*, 2009a; Huang *et al.*, 2009b). The identified genes were also jointly annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The genes that were annotated in the KEGG database as being involved in signaling pathways were subjected to further analysis. Pathways with Benjamini-adjusted p value ≤ 0.05 were considered to be statistically significant.

Integration of the miRNAs dataset with gene expression dataset and calculation of correlation were performed in MAGIA2, a web tool for the integrated analysis of target predictions, miRNA and gene expression data (Bisognin *et al.*, 2012). MiRNA target predictions include transcription factor binding sites (TFBS) within miRNA and gene

promoters. In this analysis, matched expression data matrices of significantly dysregulated miRNAs and genes (BH adjusted p value ≤ 0.05) were uploaded for integrated analysis. EntrezGene IDs and DIANA-microT (Maragkakis *et al.*, 2009) target prediction algorithms were selected. Anticorrelated expressions were investigated between miRNA and their putative target genes using Pearson correlation measure.

Results

The effect of lymph node status on survival duration of PTC patients

Overall survival in PTC patients was not influenced by LNM status (Fig. 1A); however, PTC patients with LNM has significantly shorter disease-free survival rate compared to PTC patients without LNM (Log-rank Mantel Cox test, $p = 0.0049$; Fig. 1B).

Differentially expressed miRNAs

We identified 181 miRNAs which were significantly differentially expressed in PTC LNM-P versus PTC LNN. On the other hand, 395 miRNAs were differentially expressed between PTC LNM-P and normal thyroid while 400 miRNAs were differentially expressed between PTC LNN and normal thyroid. Among the 181 miRNAs significantly expressed in PTC LNM-P versus PTC LNN, 110 were upregulated and 71 were downregulated (Supplementary Table 2). Figure 2 illustrates a heatmap representing the expression levels of 181 deregulated miRNAs in PTC LNM-P versus PTC LNN. The list of top deregulated miRNAs includes hsa-miR-146b, hsa-miR-375, hsa-miR-31, hsa-miR-7-2 and hsa-miR-204 ($\log_2$ fold change 1.7, 1.3, 1, -1.1 and -1.3, respectively, Fig. 3).

Differentially expressed genes

Initial filtering revealed 8611 significantly deregulated genes in PTC LNM-P versus PTC LNN, 14192 genes in PTC LNM-P versus normal thyroid and 13392 genes in PTC LNN versus normal thyroid. There were 4135 upregulated and 4476 downregulated genes in PTC LNM-P relative to PTC LNN. By increasing the stringency of selection to genes with $\log_2$ fold change ≥ 1 or ≤ -1, 407 genes were identified as strongly deregulated. Among the strongly deregulated genes were *SFTPB*, *CLDN10*, *DIO1* and *MT1G* ($\log_2$ fold change 3.1, 2.9, -2.2 and -2.5 respectively, Supplementary Table 4). Various cancer-related genes were also differentially expressed significantly, including *BRAF*, *BRCA2*, *VEGFA*, *VEGFB*, *RET*, *PIK3CA*, *CTNNB1* and *GNAS* (Supplementary Table 4).

Enriched pathways in PTC LNM-P

The significantly dysregulated genes in PTC LNM-P versus PTC LNN were mainly enriched in 12 KEGG pathways including oxidative phosphorylation (OxPhos), Parkinson's disease, focal adhesion, Alzheimer's disease, valine, leucine and isoleucine degradation, pathways in cancer, cell adhesion molecules (CAMs), leukocyte transendothelial migration, cytokine-cytokine receptor interaction, small cell lung cancer, Huntington's disease and extracellular matrix receptor interaction (Fig. 4A). When we overlapped the results from the three comparison groups (PTC LNM-P versus PTC LNN, PTC LNM-P versus normal thyroid and PTC LNN versus normal thyroid), four unique pathways potentially involved in metastasis to the lymph nodes were significantly enriched, namely, oxidative phosphorylation (OxPhos), cell adhesion molecules (CAMs), leukocyte transendothelial migration and cytokine-cytokine receptor interaction pathways (Fig. 4A). The oxidative phosphorylation pathway was the most significantly perturbed (p = 4.70E-06) with general downregulation of 90 OxPhos-related

genes (Fig. 5). Focal adhesion and pathways in cancer were commonly enriched in all the three group comparisons. Pathways in cancer is a collection of general cancer-related pathways and is an indication that many essential carcinogenic processes may be under the influence of dysregulated miRNAs (Pizzini *et al*., 2013). On the other hand, ECM-receptor interaction pathway and the valine, leucine and isoleucine degradation pathway were commonly enriched only in PTC LNM-P versus PTC LNN and PTC LNM-P versus normal thyroid but were not enriched in PTC LNN versus normal thyroid (Fig. 4B).

Integrated mixed regulatory circuits, involving miRNAs, genes and TFs in PTC LNM-P

To obtain a more comprehensive insight into the molecular circuits behind LNM in PTC, we focused on functional miRNA-target relationships by performing an *in silico* integration between differentially expressed miRNAs and genes using MAGIA2. Transcription factors (TFs) predicted to be regulated by or regulating our sets of differentially expressed miRNAs were experimentally validated interactions reported in the mirGen2.0 database (Friard *et al*., 2010) and TransmiR (Wang *et al*., 2010a), whereas the TF–gene interactions were acquired from the 'TFBS conserved' track of the University of California Santa Cruz (UCSC) genome annotation for humans (version hg19). Our results show that 12 miRNAs are involved in the strongest 200 interactions and they were identified as significant by MAGIA2. Hsa-miR-147b, hsa-miR-301b, hsa-miR-375, hsa-miR-496, hsa-miR-543, hsa-miR-577, hsa-miR-765, hsa-miR-892a, hsa-miR-934, hsa-miR-935, hsa-miR-940 and hsa-miR-944 were predicted to regulate and/or being regulated by 3746 genes and 1987 TFs (Fig. 6). Hsa-miR-577 and hsa-miR-147b consistently appeared in the top 20 regulatory circuits across all group comparisons. Interestingly, hsa-miR-301b appeared in both of the top 20

circuits in PTC LNM-P versus PTC LNN or normal thyroid but was absent in PTC LNN versus normal thyroid (Supplementary fig. 3).

Discussion

In this study, we explored the landscape of miRNA and mRNA expression of PTC using data obtained from the TCGA THCA project aiming to identify key pathways involved in lymph node metastasis. Our analysis revealed 110 upregulated miRNAs and 71 downregulated miRNAs in PTC LNM-P versus PTC LNN. The top deregulated miRNAs includes hsa-miR-146b, hsa-miR-375, hsa-miR-31, hsa-miR-7-2 and hsa-miR-204. Our findings are supported by several other similar studies, and in particular hsa-miR-146b, which was reported to be upregulated in PTC LNM-P versus PTC LNN (Lee *et al*., 2013; Yang *et al*., 2013; Acibucu *et al*., 2014; Deng *et al*., 2015).

Hsa-miR-146 is one of the widely studied miRNAs in thyroid cancers and has been shown to be frequently upregulated in PTC (He *et al*., 2005; Pallante *et al*., 2006; Tetzlaff *et al*., 2007; Chen *et al*., 2008; Yip *et al*., 2011; Chou *et al*., 2010; Chou *et al*., 2013; Sun *et al*., 2013), anaplastic thyroid cancer (Fassina *et al*., 2014) and follicular thyroid cancer (FTC) (Wojtas *et al*., 2014). Functional analyses of hsa-miR-146 revealed its involvement in various cellular functions including migration, invasion, proliferation, colony-forming ability, cell cycle, and resistance to chemotherapy-induced apoptosis in *BRAF*-mutated cell lines (Chou *et al*., 2013; Deng *et al*., 2015; Geraldo *et al*., 2012). Using multivariate logistic regression analysis, Chou and colleagues demonstrated that increased hsa-miR-146b expression is one of the independent risk factors for poor prognosis in PTC, implicating the potential of this miRNA as a prognostic marker (Chou *et al*., 2013).

The genes targeted by hsa-miR-146b are mostly unknown, and to date there are only two genes which has been reported as the direct targets of this miRNA in PTC. Geraldo *et al.* (2012) reported *SMAD4*, an important member of the transforming growth factor β (TGF-β) signaling pathway, as the target of hsa-miR-146b-5p (Geraldo *et al*., 2012). The direct binding of hsa-miR-146b-5p on the *SMAD4* UTR was confirmed via a luciferase reporter assay and the inhibition of hsa-miR-146b-5p expression resulted in significantly increased SMAD4 gene and protein expression levels in the human PTC cell lines. Furthermore, the inhibition of hsa-miR-146b-5p increased the cellular response to the TGF-β anti-proliferative signal, leading to significant reduction of cell proliferation (Geraldo *et al*., 2012). In a more recent study, the Zinc Ring Finger 3 (ZNRF3) gene was revealed as a direct target of hsa-miR-146b-5p and this miRNA was shown to stimulate cell migration, invasion and epithelial-to-mesenchymal transition (EMT) by downregulating *ZNRF3* (Deng *et al*., 2015). Another study showed that *ZNRF3* inhibits Wnt signaling by interacting with FZD and LRP 5/6 complexes, hence promoting Wnt receptor ubiquitination and degradation (Hao *et al*., 2012). Hsa-miR-146b-5p increases the cell surface levels of FZD6 and LRP6 via suppression of *ZNRF3*, causing enhanced Wnt/β-catenin signaling. These findings revealed a novel mechanism of hsa-miR-146b-5p in mediating the induction of EMT and implied the role of *ZNRF3* as a tumor suppressor in PTC (Deng *et al*., 2015). Additional efforts to identify genes regulated by hsa-miR-146b associated with LNM will eventually revealed new biomarkers that can be utilized to correlate with disease outcome in PTC patients.

Hsa-miR-204 expression in PTC LNM-P is significantly lower than in PTC LNN. This is the first report showing the downregulation of hsa-miR-204 in PTC LNM-P. This miRNA was also downregulated in PTC compared to adjacent normal thyroid tissue and noncancerous thyroid (Swierniak *et al*., 2013). It is likely that hsa-miR-204 is downregulated in PTC compared to normal or benign thyroid disease and is further supressed when lymph node

metastasis occurs. This miRNA is known as a tumor suppressor miRNA and is downregulated in various cancers including renal clear cell carcinoma (Gowrishankar *et al*., 2014), minimal deviation adenocarcinoma (MDA) of uterine cervix (Lee *et al*., 2014) and breast cancer (Li *et al*., 2014). This miRNA has also been shown to have a prognostic value; low level of hsa-miR-204-5p expression was correlated with LNM, advanced stage and low survival rate in endometrial cancer (Bao *et al*., 2013), and also poor prognosis in colorectal cancer (Yin *et al*., 2014). *In vitro* functional analyses revealed the involvement of hsa-miR-204 in inhibiting the clonogenic growth, migration and invasion of endometrial carcinoma cells (Bao *et al*., 2013). In addition, restoration of hsa-miR-204-5p expression supressed cell proliferation, migration, invasion and induced apoptosis and chemotherapeutic sensitivity in colorectal cancer cell (Yin *et al*., 2014).

The validated targets for hsa-miR-204 in PTC are also not well-characterized. To date there is only one study investigating the functional role of hsa-miR-204 in PTC (Liu *et al*., 2015). Enforced expression of hsa-miR-204-5p inhibited cell proliferation and induced apoptosis and cell cycle arrest in PTC cell lines (TCP-1 and BCPAP). In addition, hsa-miR-204-5p also inhibits PTC cell tumorigenicity *in vivo* (Liu *et al*., 2015). Bioinformatics prediction analyses using three algorithms (miRanda, Pictar, and TargetScan) revealed the insulin-like growth factor-binding protein 5 (IGFBP5), a gene playing an essential role in carcinogenesis (Beattie *et al*., 2006), as a potential target of hsa-miR-204-5p. Luciferase reporter assay confirmed the direct binding of hsa-miR-204-5p to the 3′ UTR of *IGFBP5* (Liu *et al*., 2015). In the same study, hsa-miR-204-5p and *IGFBP5* expression were also shown to be inversely correlated. Their findings confirmed the role of hsa-miR-204-5p as a tumor suppressor in PTC and revealed the potential use of this miRNA as a therapeutic agent in the treatment of PTC.

In an attempt to identify genes and pathways associated with mortality in PTC, Nilubol et al. (2011) performed genome-wide expression (GWE) analysis in 64 PTC patients and identified the oxidative phosphorylation pathway as one of the significantly perturbed pathways. In addition, Lee and colleagues also showed that the expression of OxPhos gene sets was significantly lower in primary PTC than in matched normal thyroid tissue (Lee et al., 2015a). Our findings revealed a similar trend with OxPhos genes being significantly downregulated in PTCs versus normal thyroid tissues as well as in PTC LNM-P versus PTC LNN. However, significant enrichment of OxPhos pathway was only observed in PTC LNM-P compared to PTC LNN. Alteration in metabolic processes has been considered as an indispensable component of malignant transformation (Lee et al., 2015a) thus the involvement of oxidative phosphorylation in LNM in PTC necessitates further investigation.

Oxidative phosphorylation is a process whereby an adenosine triphosphate (ATP) is produced as a result of electrons transfer from nicotinamide adenine dinucleotide (NADH) or flavin-adenine dinucleotide ($FADH_2$) to oxygen by a series of electron carriers (Berg et al., 2002). The thyroid gland is an endocrine organ with a high energy consumption and oxidative processes are crucial for thyroid hormone synthesis (Lee et al., 2015b). The mitochondria is responsible for providing 90% of the cellular energy necessary for various biological functions through oxidative phosphorylation and plays an important role in energy metabolism in the normal thyroid gland and in thyroid tumors (Kim et al., 2012). The mitochondria affects many cell signaling pathways by playing crucial roles in apoptosis, cell proliferation and cellular Ca^{2+} homeostasis (Rustin 2002). Mitochondrial DNA (mtDNA) content was shown to be higher in PTC compared to the paired normal DNA and in normal controls (Mambo et al., 2005). Despite advancement in the elucidation of molecular events underlying thyroid carcinogenesis in the last decade, the function and nature of energy metabolism in thyroid cancer remain unclear (Lee et al., 2015b).

In addition to oxidative phosphorylation, we also identified significant enrichment of other cancer-related pathways such as cell adhesion molecules (CAMs), leukocyte transendothelial migration and cytokine-cytokine receptor interaction pathways which were unique to PTC LNM-P versus PTC LNN. Interestingly, these pathways were not significantly enriched when PTCs (LNM-P and LNN) were compared to normal thyroid tissues. Cell adhesion is a vital feature in cell migration, growth and differentiation in vertebrate cells (Okegawa et al., 2004). CAMs have been implicated in a wide variety of cellular functions including apoptosis, signal transduction, cellular communication, as well as inflammatory and immune responses (Okegawa et al., 2004). For metastasis to happen, the cancer cells must enter the bloodstream or lymphatic circulation, which probably involves the degradation of intercellular adhesion thus making CAMs the probable contributors in the development of metastatic disease (Hanahan and Weinberg 2011). CAMs are likely to be involved too in transendothelial migration which is also required for metastasis to occur. Transendothelial migration is a biological process that comprises of continuous breaking and reconstructing of intercellular contacts and is accompanied by alterations in cell shape and cytoskeletal reorganization in the tumor cell and its adjacent endothelial cells (Voura et al., 1998). It occurs via diapedesis, whereby the cells move in an ameboid manner through compactly adjoining endothelial borders and/or through the endothelial cell itself (Muller 2011). We found that the leukocyte transendothelial migration pathway was significantly enriched in PTC LNM-P versus PTC LNN. In addition, the expression levels of genes involved in cytokine-cytokine receptor interaction were also significantly altered in PTC LNM-P versus PTC LNN. Cancer cells are known to express cytokine receptors on their cell surface (Koji et al., 2002). Cytokine receptors are glycoproteins that bind precisely to cytokines and transduce their signals. These receptors permit cells to communicate with the extracellular environment by reacting to signals produced in the environs or in other parts of the organism (Bagley et al., 1997). Therefore, the

first binding of cytokines to their receptors is a main event that occurs quickly, at very low cytokine concentrations, is usually irrevocable, and leads to intracellular alterations resulting in a biologic response (Bagley et al., 1997). The biological response can differ between cytokine receptors and from cell to cell but generally it includes gene expression, cell cycle and discharge of mediators such as cytokines themselves (Bagley et al., 1997). Taken together, it could be hypothesized that metastasis to the lymph node in PTC occurred via changes in oxidative phosphorylation, CAMs, leukocyte transendothelial migration and cytokine-cytokine receptor interaction. However, some pathways in our analysis, such as valine, leucine and isoleucine degradation, could not be associated with oncogenesis or metastasis and may need further investigation.

Our integrated analysis revealed hsa-miR-301b's presence in the top 20 circuits in both PTC LNM-P versus PTC LNN and PTC LNM-P versus normal thyroid but was absent in PTC LNN versus normal thyroid despite significant downregulation with modest fold change (log_2 fold change of -0.3). Hsa-miR-301 is located in the intronic region of *SKA2* (spindle and kinetochore associated complex subunit 2) and belongs to the hsa-miR-130 microRNA precursor family (Chao et al., 2010). In contrast to our findings, hsa-miR-301 upregulation has been reported in various cancers of non-thyroid origins and given that a miRNA can act either as an oncomiR or tumor suppressor depending on the cellular context and tissue type (Garzon et al., 2009), this observation is not unexpected. There is no evidence of hsa-miR-301b dysregulation in PTC so far, but it was reported to be upregulated in follicular thyroid adenoma compared to normal thyroid tissue (Rossing et al., 2012). It is also upregulated in CRC without LNM in comparison to paracancerous control (Wang et al., 2010b). The inhibition of hsa-miR-301 decreased breast cancer cell proliferation, clonogenicity, migration, invasion, tamoxifen resistance, tumor growth and microvessel density, further establishing this miRNA as an

oncomiR (Shi et al., 2011). *FOXF2*, *BBC3*, *PTEN*, and *COL2A1* were confirmed as its direct targets through luciferase reporter assays (Shi et al., 2011).

Transcription factors (TFs) are a group of proteins involved in the initiation of transcription and are important for the regulation of genes. Majority of oncogenes and tumor suppressor genes encode the TFs (Ell and Yang 2013). Dysregulation of oncogenic or tumor suppressive TFs could influence multiple steps of the metastasis cascade, leading to cancer progression (Ell and Yang 2013). The involvement of TFs in PTC has been investigated since decades ago and several thyroid-specific TFs have been identified (Guazzi et al., 1990; Fabbro et al., 1994). Most recently, the glioma-associated oncogene homolog 1 (GLI1) has been identified as a TF marker for LNM in PTC and it affects tumor aggressiveness via the Hedgehog signaling pathway (Lee et al., 2015c). The hepatic leukemia factor (HLF) is the only TF which appeared in the top 20 circuits of PTCs with or without LNM versus normal thyroid from our integrated analysis. On the other hand, REL was identified in the top 20 circuits only in PTC LNM-P in comparison to PTC LNN and will be discussed further in the following section.

The HLF is a transcription factor that facilitates thyroid hormone activation from the thyroid hormone receptor/retinoid X receptor heterodimer to hypoxia-inducible factor (HIF-1α) (Otto and Fandrey 2008). Triiodothyronine (T3) indirectly increases HIF-1α mRNA by increasing the expression of HLF, subsequently initiating the transcription of HIF-1α transcription factor (Burrows et al., 2011). HIF is another transcription factor which acts under hypoxia and thus is active in a number of diseases associated with low oxygen environment including cancer (Burrows et al., 2011). In fact, the HIF-1α protein was differentially expressed in primary thyroid cancers associated with advanced stage; its expression was supressed in normal thyroid tissue and was highest in the most aggressive dedifferentiated anaplastic thyroid

carcinomas (ATCs) (Hanada et al., 2004), supporting its role for thyroid tumor aggressiveness, progression as well as metastasis.

The REL or NF-kappaB (NFkB) proteins are a family of structurally-related eukaryotic transcription factors involved in the regulation of various normal cellular processes including immune and inflammatory responses, developmental processes, cellular growth as well as apoptosis (Caamaño and Hunter 2002). Hence, REL/NFkB TFs are active in many disease conditions including thyroid cancer (Pacifico and Leonardi 2010). Mutation in the oncogene which is involved in thyroid carcinogenesis such as *BRAF* V600E, is able to induce activation of NFkB in PTC *in vitro* (Palona et al., 2006). In our study we identified the significant involvement of REL/NFkB in lymph node metastasis of PTC which is in concordance with published data (Du et al., 2006).

Conclusions

In summary, we found evidence of five miRNAs differentially expressed in PTC LNM-P. Enrichment analysis revealed that alteration in oxidative phosphorylation pathway could be a key event involved in the lymph node metastasis of PTC suggesting that manipulation of the energy metabolism processes may provide an alternative therapeutic target for tackling metastasis or recurrence. In addition, via the integrated analysis we discovered that hsa-miR-301b might be involved in regulating LNM in PTC via interactions with HLF, HIF and REL/NFkB. As far as we know, the roles of these TFs have been explored in PTC; however the exact roles of this miRNA with these TFs in LNM in PTC have not been studied. Hence, further investigation is necessary for future research in order to completely unravel the mechanism of LNM in PTC.

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685

Table 1(on next page)

Patient Characteristics and Integrated Profiles in the TCGA PTC Cohort

1 Table 1. Patient Characteristics and Integrated Profiles in the TCGA PTC Cohort

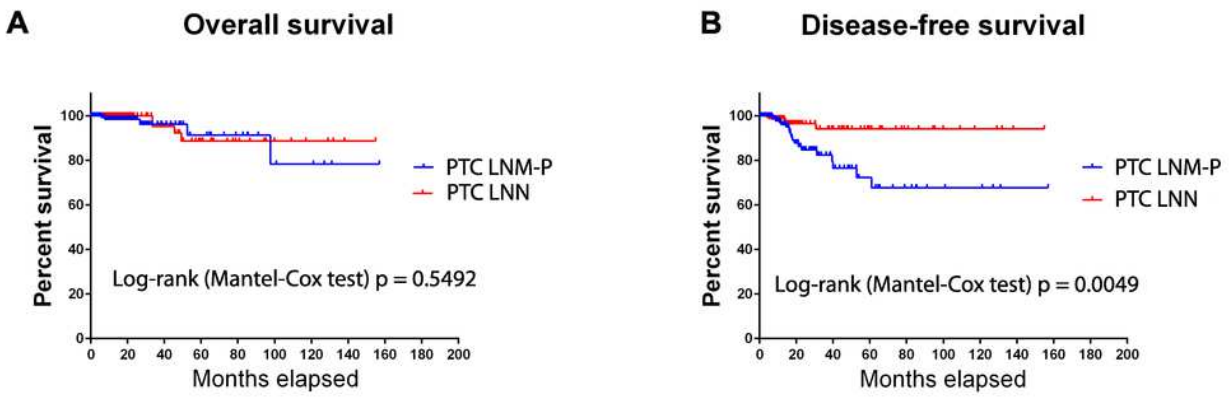
Variables	**PTC LNN**	**PTC LNM-P**		
	N0 (n = 213)	**N1 (n = 53)**	**N1a (n = 86)**	**N1b (n = 66)**
Age range (years)	15 - 85	19 - 83	18 - 83	19 - 89
Mean age	49.4	41.9	43.5	48.4
Gender (n)				
Male	50 (23.5%)	14 (26.4%)	25 (29.1%)	27 (40.9%)
Female	163 (76.5%)	39 (73.6%)	61 (70.9%)	39 (59.1%)
Disease free status				
Recurred/progressed	5 (2.3%)	7 (13.2%)	6 (7%)	6 (9.1%)
Disease free	178 (83.6%)	41 (77.4%)	75 (87.2%)	47 (71.2%)
Unknown	30 (14.1%)	5 (9.4%)	5 (5.8%)	13 (19.7%)
Disease free (range in months)	0.03 - 155	0 - 131	0 - 157	0.2 – 46
Mean disease free survival	23.6 (n = 183)	34.5 (n = 48)	21.5 (n = 81)	13.5 (n = 53)
Overall survival status				
Deceased	35 (16.4%)	12 (22.6%)	11 (12.8%)	19 (28.8%)
Alive	178 (83.6%)	41 (77.6%)	75 (87.2%)	47 (71.2%)
Overall survival (range in months)	0.03 - 155	0 - 131	0 - 157	0.2 – 97.7
Mean overall survival	24.3 (n = 182)	35.2 (n = 43)	21.4 (n = 75)	15.2 (n = 50)
Extrathyroidal extension				
None	160 (75.1%)	31 (58.5%)	49 (57%)	37 (56.1%)
Minimal (T3)	42 (19.7%)	14 (26.4%)	33 (38.4%)	23 (34.8%)
Moderate/ advanced (T4a)	3 (1.4%)	5 (9.4%)	1 (1.2%)	4 (6.1%)
Very advanced (T4b)	0 (0%)	1 (1.9%)	0 (0%)	0 (0%)
Unknown	8 (3.8%)	2 (3.8%)	3 (3.5%)	2 (3%)
BRAF status				
Mutated	94 (44.1%)	25 (47.2%)	53 (61.6%)	32 (48.9%)
Wild type	119 (55.9%)	28 (52.8%)	33 (38.4%)	34 (51.5%)

2

3

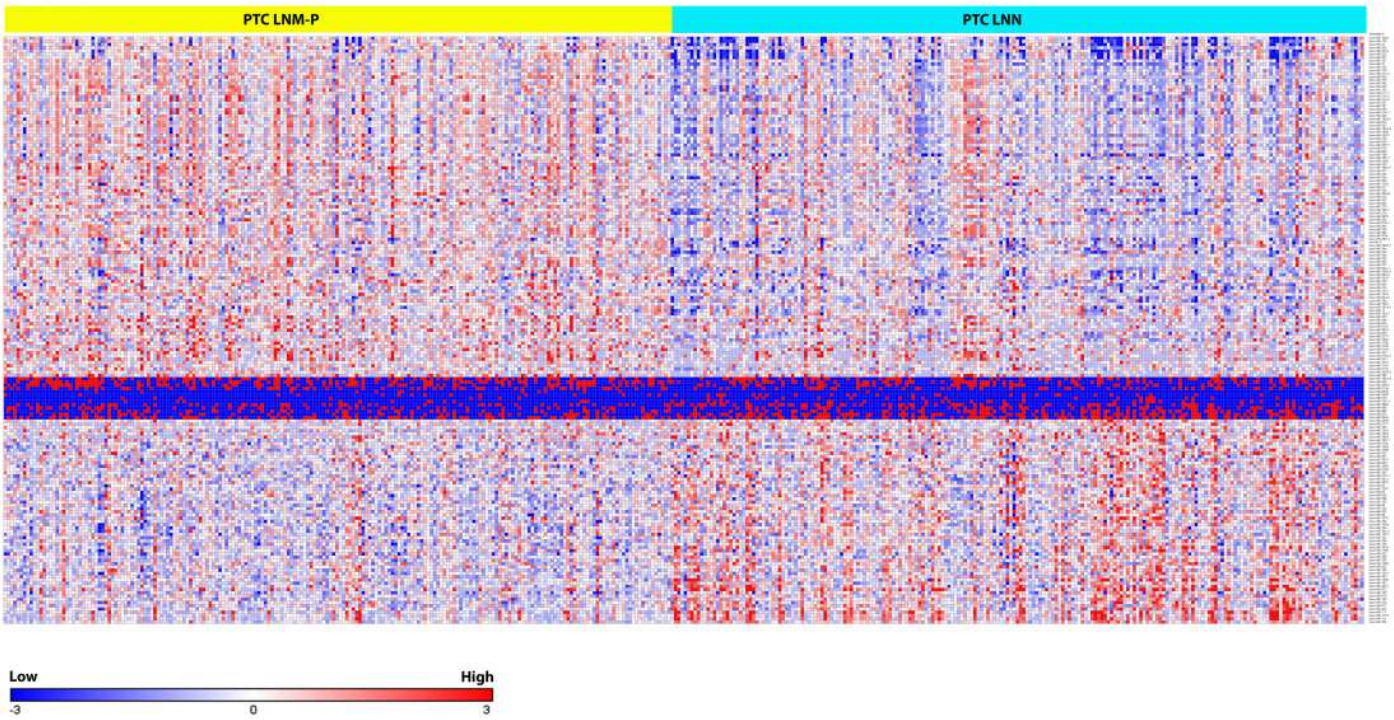
1

Survival analysis of PTC with LNM and PTC without LNM



2

Heat Map of the 181 Differentially Expressed miRNAs in PTC LNM-P and LNN (Student's T-test with BH corrected p value ≤0.05).

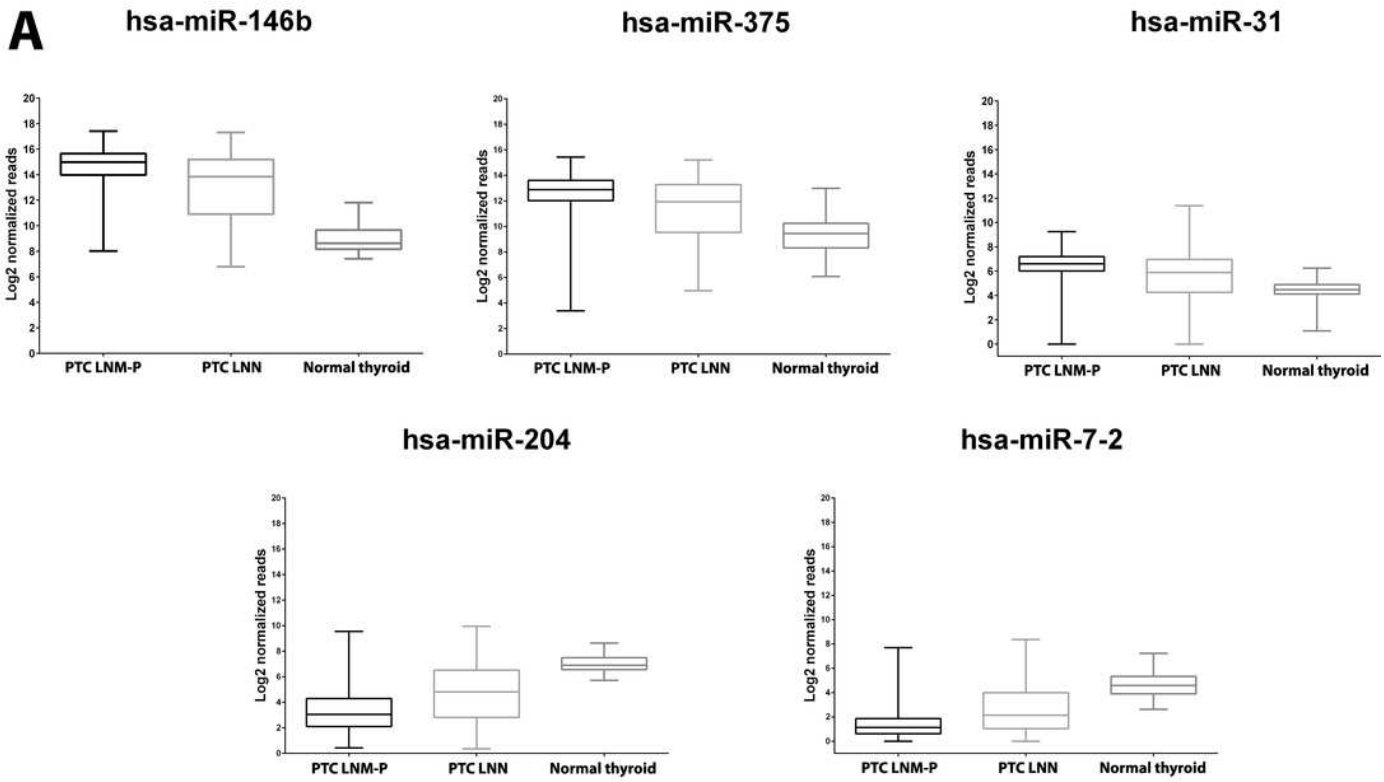


3

Expression levels of five selected miRNAs deregulated in PTC.

Boxplots (A) illustrate log2 normalized miRNA reads in PTC LNM-P, PTC LNN and normal thyroid. Table (B) showing log2 fold change and p value of selected miRNAs in PTC LNM-P compared to PTC LNN.

A



B

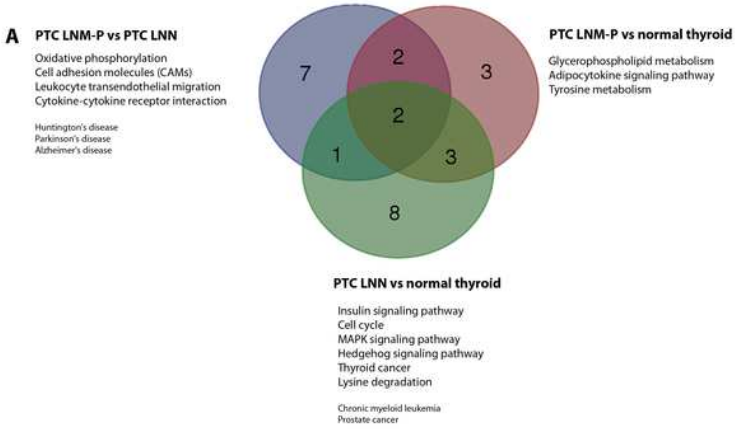
miRNA ID	Log_2 fold change	BH adj. p value
hsa-miR-146b	1.7	1.41E-03
hsa-miR-375	1.4	3.00E-02
hsa-miR-31	1.0	4.59E-09
hsa-miR-7-2	-1.1	3.00E-02
hsa-miR-204	-1.4	2.11E-05

Student's T-test with BH corrected p-value ≤ 0.05; log_2 fold change ≥ 1 or ≤ -1.

4

Significantly enriched pathways in PTCs.

Significant KEGG pathway associations to 8611 significantly deregulated genes in PTC LNM-P versus PTC LNN, 14192 genes in PTC LNM-P versus normal thyroid and 13392 genes in PTC LNN versus normal thyroid.



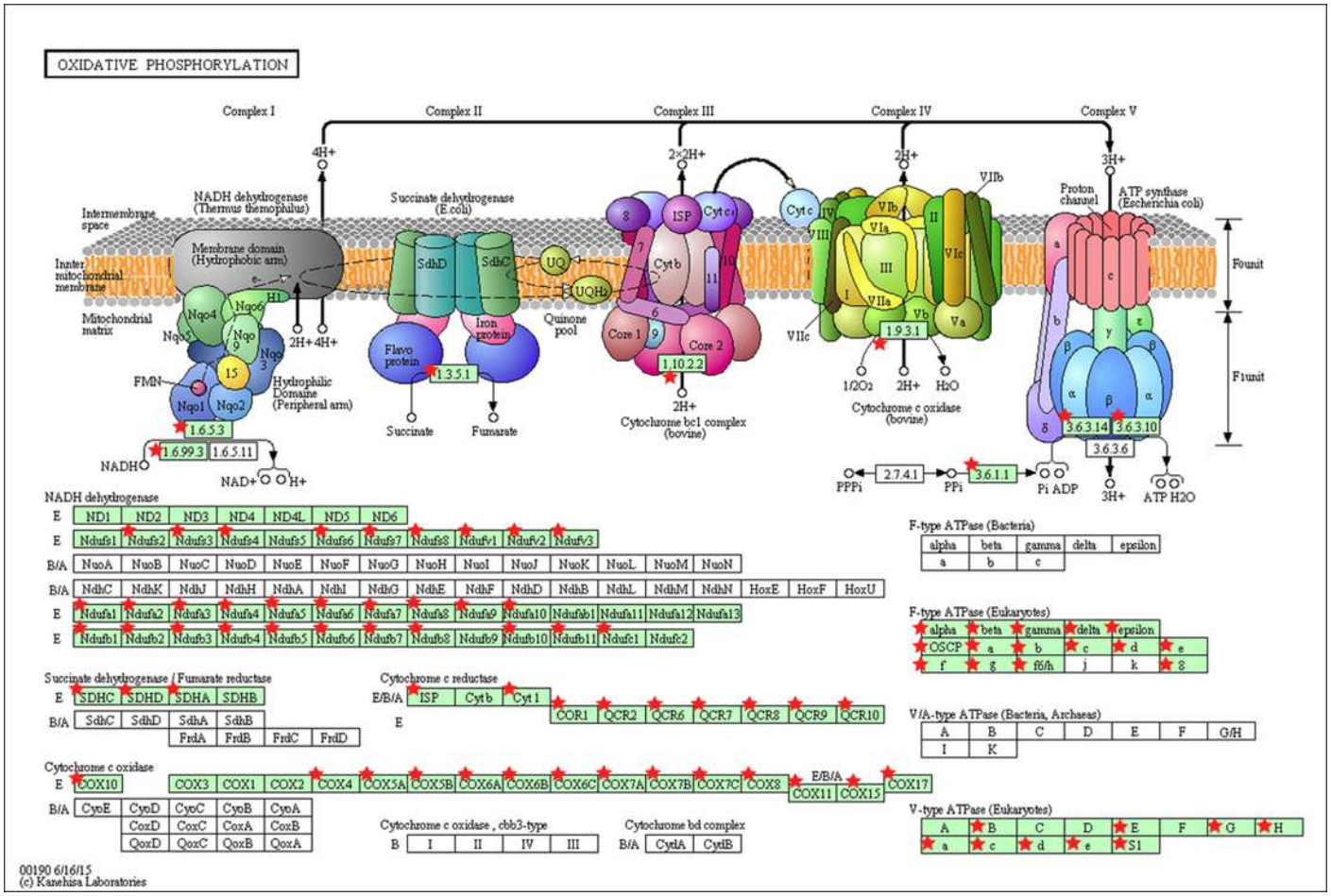
B

Group comparison	Enriched pathway	Benjamini adj. p value
PTC LNM-P vs PTC LNN	Oxidative phosphorylation	4.70E-06
	Parkinson's disease	5.80E-06
	Focal adhesion	3.40E-05
	Alzheimer's disease	4.80E-05
	Valine, leucine and isoleucine degradation	5.50E-03
	Pathways in cancer	6.00E-03
	Cell adhesion molecules (CAMs)	7.40E-03
	Leukocyte transendothelial migration	2.00E-02
	Cytokine-cytokine receptor interaction	1.90E-02
	Small cell lung cancer	1.90E-02
	Huntington's disease	1.90E-02
	ECM-receptor interaction	3.30E-02
PTC LNM-P vs normal thyroid	ECM-receptor interaction	8.70E-03
	PPAR signaling pathway	4.60E-03
	p53 signaling pathway	4.00E-03
	Pathways in cancer	5.50E-03
	Focal adhesion	6.80E-03
	Valine, leucine and isoleucine degradation	2.10E-02
	Axon guidance	3.50E-02
	Glycerophospholipid metabolism	4.60E-02
	Adipocytokine signaling pathway	5.10E-02
	Tyrosine metabolism	4.70E-02
PTC LNN vs normal thyroid	Axon guidance	8.10E-04
	Pathways in cancer	2.50E-03
	Insulin signaling pathway	5.30E-03
	Cell cycle	7.70E-03
	MAPK signaling pathway	8.10E-03
	Hedgehog signaling pathway	8.90E-03
	Chronic myeloid leukemia	8.80E-03
	p53 signaling pathway	1.80E-02
	Focal adhesion	2.80E-02
	PPAR signaling pathway	2.90E-02
	Thyroid cancer	3.40E-02
	Prostate cancer	3.30E-02
	Small cell lung cancer	4.30E-02
	Lysine degradation	4.10E-02

5

KEGG pathway map illustrating oxidative phosphorylation in human.

The OxPhos-related genes significantly altered in PTC LNM-P compared to PTC LNN were depicted with red star.



6

Grand view of regulatory circuits constructed using significantly dysregulated miRNAs and genes in PTC LNM-P compared to PTC LNN.