A Metabolic Phenotype Based on Mitochondrial Ribosomal Protein Expression as a Predictor of Lymph Node Metastasis in Papillary Thyroid Carcinoma

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Abstract: Metabolic reprogramming has been regarded as an essential component of malignant transformation. However, the clinical significance of metabolic heterogeneity remains poorly characterized. The aim of this study was to characterize metabolic heterogeneity in thyroid cancers via the analysis of the expression of mitochondrial ribosomal proteins (MRPs) and genes involved in oxidative phosphorylation (OxPhos), and to investigate potential prognostic correlations. Gene set enrichment analysis (GSEA) verified by reverse transcription polymerase chain reaction and gene network analysis was performed using public repository data. Cross-sectional observational study was conducted to classify papillary thyroid cancer (PTC) by the expression of MRP L44 (MRPL44) messenger RNA (mRNA), and to investigate the clinicopathological features. GSEA clearly showed that the expression of OxPhos and MRP gene sets was significantly lower in primary thyroid cancer than in matched normal thyroid tissue. However, 8 of 49 primary thyroid tumors (16.3%) in the public repository did not show a reduction in OxPhos mRNA expression. Remarkably, strong positive correlations between MRPL44 expression and those of OxPhos and MRPs such as reduced nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) 1 α subcomplex, 5; succinate dehydrogenase complex, subunit D; cytochrome c, somatic; adenosine triphosphate synthase, H+ transporting, mitochondrial Fo complex, subunit C1 (subunit 9); and MRP S5 (MRPS5) (P < 0.0001) were clearly denoted, suggesting that MRPL44 is a representative marker of OxPhos and MRP expression. In laboratory experiments, metabolic heterogeneity in oxygen consumption, extracellular acidification rates (ECARs), and amounts of OxPhos complexes were consistently observed in BCPAP, TPC1, HTH-7, and XTC.UC1 cell lines. In PTCs, metabolic phenotype according to OxPhos amount defined by expression of MRPL44 mRNA was significantly related to lymph node metastasis (LNM) (P < 0.001). Furthermore, multivariate analysis clearly indicated that expression of MRPL44 is associated with an increased risk of lateral neck LNM (odds ratio 9.267, 95% confidence interval 1.852–46.371, P = 0.007). MRPL44 expression may be a representative marker of metabolic phenotype according to OxPhos amount and a useful predictor of LNM.

INTRODUCTION

Papillary thyroid cancer (PTC) is the most common endocrine malignancy whose incidence is rapidly increasing worldwide. However, the treatment strategy of PTC is controversial because patients with PTC have widely different prognoses. Nonetheless, the clinical applications of these markers to predict the presence of poor prognostic factors such as extrathyroidal extension (ETE) or lymph node metastasis (LNM) to characterize persistent or recurrent PTC at initial diagnosis. In this regard, the v-Raf murine sarcoma viral oncogene homolog B (BRAF) V600E mutation, galectin-3, and human mesothelial cell-1 are all considered potential candidates for the prediction of prognosis. Nonetheless, the clinical applications of these molecular markers are still limited. Metabolic reprogramming is an essential component of malignant transformation. Following the discovery of the Warburg effect, cancer cells were shown to produce energy predominantly through higher glycolytic activity (glycolytic phenotype) rather than through oxidative phosphorylation.
(OxPhos) using the electron transport chain (ETC). However, in addition to aerobic glycolysis (Warburg effect), glutamine addiction, somatic isocitrate dehydrogenase mutations, glycine-serine addiction, and the reverse Warburg effect suggested the existence of diverse metabolic phenotypes in cancer cells. In addition, cancer cells generating cellular glycine/serine addiction, and the reverse Warburg effect for predicting poor prognosis and planning the extent of surgery. Of LNM, which suggests that it may be a useful clinical marker of MRP L44 (MRPL44) messenger RNA (mRNA). Interest-
Isolation of Mitochondria and BN-PAGE

Isolation of mitochondria and blue native (BN) BN-PAGE was performed as described previously using the NativePAGE Novex Bis-Tris Gel system (Invitrogen). B Briefly, cells were homogenized in isolation buffer B (210 mM Mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.2) with a Teflon-glass homogenizer. The homogenate was then centrifuged at 600 × g for 10 minutes at 4°C, and the resulting supernatant was recentrifuged at 17,000 × g for 10 minutes at 4°C. The mitochondrial fractions, which were recovered in the pellet, were washed with buffer B and resuspended in the same buffer. Mitochondria were either used immediately or stored at −80°C for later use. Isolated mitochondria (50 μg) were solubilized using NativePAGE sample buffer with 0.5% n-dodecyl-β-maltoside or 1% digitonin (Invitrogen). The suspensions were centrifuged at 20,000 × g for 10 minutes at 4°C. The resulting supernatants were loaded onto a NativePAGE Novex 3% to 12% Bis-Tris gel (Invitrogen). After the run was complete, the gel was transferred to a PVDF membrane using the iBlot Gel Transfer System (Invitrogen). The membrane was fixed with 8% acetic acid. After overnight drying, the membrane was destained with methanol. To detect the OxPhos complex, the Mitoprofile Total OXPHOS Rodent WB Antibody Cocktail, Abcam, Cambridge, MA, USA (Mitosciences/Abcam) was used. After incubation in the primary antibody dilution, the membrane was washed and visualized using the WesternBreeze Chromogenic Western Blot Immunodetection Kit (Invitrogen).

RESULTS

Metabolic Heterogeneity According to mRNA Expression of OxPhos Proteins and MRPs in PTC

To confirm the heterogeneity of mRNA expression of OxPhos and MRPs in PTC, we performed GSEA using public repository data. In the analysis using GSE33630 (n = 105), both the OxPhos gene set (P = 0.0395, false discovery rate (FDR) q value = 0.225, Figure 1A) and the MRP gene set (P = 0.0022, FDR q value = 0.001, Figure 1B) showed statistically significant enrichment in normal thyroid tissues. In addition, in the analysis using GSE6004 (n = 18), the OxPhos and MRP gene sets were consistently enriched in normal thyroid tissues (P = 0.0388, FDR q value < 0.05; P = 0.002, FDR q value < 0.01, respectively; Supplementary Figure 1A and B, http://links.lww.com/MD/A138). In agreement with the Warburg effect (aerobic glycolysis), GSEA clearly indicated a reduction in OxPhos and MRP gene sets, suggesting that cancer cells from both papillary and anaplastic thyroid cancers (ATC) predominantly produce energy via a high rate of glycolysis. However, through careful review of the raw data in GSEA (GSE33630), we found that 8 of 49 PTC cases (16.3%) did not show a reduction in OxPhos mRNA expression (Supplementary Figure 2, http://links.lww.com/MD/A138).

![Figure 1](http://links.lww.com/MD/A138)

**Figure 1.** GSEA using public repository data (GSE336300). (A) GSEA for KEGG, OxPhos pathway gene set. (B) GSEA for KEGG, mitochondrial ribosomal pathway gene set. ATC = anaplastic thyroid cancers, FDR = ??, GSEA = gene set enrichment analysis, OxPhos = oxidative phosphorylation, PTC = papillary thyroid cancer.
MD/A138). In addition, the HPA program (used to explore the entire human proteome using an antibody-based approach funded by the nonprofit organization Knut and Alice Wallenberg Foundation) indicated that expression of MRPL44 was not decreased in all PTCs. As shown in Supplementary Figure 3A, http://links.lww.com/MD/A138, normal thyroid follicular cells showed moderate MRPL44 staining, whereas PTC cells showed low (Supplementary Figure 3B, http://links.lww.com/MD/A138) to moderate (Supplementary Figure 3C, http://links.lww.com/MD/A138) staining, suggesting heterogeneous expression of MRPs in PTCs. In addition, the staining intensity of NDUFA5 was also moderate to strong in PTC (Supplementary Figure 4, http://links.lww.com/MD/A138), was also moderate in normal thyroid follicular cells, and was decreased in all PTCs. As shown in Supplementary Figure 3A, http://links.lww.com/MD/A138, normal thyroid follicular cells showed moderate MRPL44 staining, whereas PTC cells showed low (Supplementary Figure 3B, http://links.lww.com/MD/A138) to moderate (Supplementary Figure 3C, http://links.lww.com/MD/A138) staining, suggesting heterogeneous expression of MRPs in PTCs. In addition, the staining intensity of NDUFA5 was also moderate to strong in PTC (Supplementary Figure 4, http://links.lww.com/MD/A138). To further investigate the observed intertumor heterogeneity of OxPhos protein and MRP expression, we analyzed the correlation between mRNA expression of OxPhos and MRP genes using the raw data from GSE33630. Interestingly, expression of MRP mRNA showed a statistically significant correlation with OxPhos mRNA expression (Figure 2A and B and Supplementary Figure 5, http:// links.lww.com/MD/A138). To further investigate the expression pattern of OxPhos and MRP mRNA expression. Accordingly, MRPs such as MRPL44 may be useful as indicators of the expression pattern of OxPhos and MRPs.

### Metabolic Phenotype in Thyroid Cancer Cell Lines

Considering the differential expression of OxPhos and MRP genes, we postulated that a certain degree of “metabolic diversity” exists in PTCs. To investigate this possibility, we

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**FIGURE 2.** Correlation between OxPhos and MRP gene expression. (A) The relationship between NDUFA5 mRNA expression and MRPL44, MRPL56, and MRPS28 expressions in GSE336300. (B) The relationship between SDHD mRNA expression and MRPL44, MRPL56, and MRPS28 expressions in GSE336300. Statistical analysis was carried out using GraphPad Prism. (C–E) Gene network analysis of the relationship between Mrpl44 expression and Atp5g2 (C), Ckmt (D), and Mrps5 (E) expressions using GSE16780 UCLA Hybrid MDP Liver Affy HT M430A (Sep11) RMA. Atp5g2 = ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (subunit 9) (Atp5g2) (Figure 2C and Supplementary Figure 6B, http://links.lww.com/MD/A138), mitochondrial creatine kinase (Ckmt, Figure 2D and Supplementary Figure 6C, http://links.lww.com/MD/A138), and Mrps5 (Figure 2E and Supplementary Figure 6D, http://links.lww.com/MD/A138). Taken together, these data indicated significant intertumor heterogeneity in OxPhos and MRP mRNA expression.
estimated OCR and ECAR in HELA cells and thyroid cancer cell lines using an XF analyzer. As shown in Figure 3A and B, HELA cells showed a higher OCR and a lower ECAR compared with most thyroid cancer cell lines, suggesting that they generate more ATP via OxPhos (oxidative phenotype) than via glycolysis. BCPAP cells, a BRAFV600E positive PTC cell line, showed a decreased OCR and the highest ECAR, which was in agreement with the higher levels of glycolysis (glycolytic phenotype) in this cell line. TPC1 cells, a PTC cell line harboring a RET/PTC1 rearrangement, had relatively lower levels of both OCR and ECAR, suggesting that this cell line generated its ATP via both glycolysis and OxPhos (combined phenotype). XTC.UC1 cells, derived from a Hurthle cell cancer harboring mutations in mitochondrial DNA (mtDNA), had a lower OCR and a higher ECAR, as expected (glycolytic phenotype). In BN-PAGE, HELA cells had the most abundant OxPhos complexes (Figure 3C), whereas complex IV was barely detectable in TPC1 cells (Figure 3C, lower panel). Consistent with the results obtained using the XF analyzer, HTH-7 cells contained more intact OxPhos complexes than TPC1 cells. Thus, the thyroid cancer cell lines displayed substantial metabolic diversity. The classification of thyroid cancer cell lines according to metabolic phenotype is shown in Figure 3D.

**Heterogeneous Expression of OxPhos and MRPs in PTC**

Based on the results obtained using public repository data, RT-PCR was performed using mRNA from primary PTC and matched normal tissues to determine the expression status of OxPhos and MRP genes. First, 12 patients whose preoperative [18F]-fluorodeoxyglucose positron emission tomography (18F-FDG PET) imaging was available were analyzed. Ten of the 12 cases showed a reduction in the expression of OxPhos and MRP genes, including NDUFB3, NDUFAS, SDHD, CYC1, CYCS, COX6A1, COX7B, ATP5G1, MRPL44, MRPL45, MRPS5, MRPS11, MRPS28, and MRPS31. However, 3 cases showed no change in the mRNA expression of OxPhos and MRP genes compared with matched normal thyroid tissues such as a case no. 4 as shown in Figure 4A. Interestingly, 18F-FDG PET imaging indicated that PTCs in all 12 cases had a nodular lesion in the thyroid gland with increased uptake of 18F-FDG (Figure 4B), suggesting high glycolytic activity. RT-PCR and 18F-FDG PET imaging data indicated that the expression of OxPhos and MRP mRNAs was heterogeneous, although glycolytic activity was still higher in PTCs than in normal thyroid tissue.

**Correlation Between MRPL44 mRNA Expression and Regional LNM**

To understand the clinical implications of metabolic diversity in PTC, we analyzed potential correlations between expression of MRPL44 mRNA and clinicopathological parameters.
Based on the heterogeneous expression of MRPL44 and NDUFA5, RT-PCR for MRPL44 was performed in 103 primary PTC and matched normal thyroid tissue. Of these, 87 PTCs (84.5%) showed a reduction in MRPL44 expression compared with that in normal tissue, whereas 16 PTCs (15.5%) showed no differences in MRPL44 mRNA expression between tumoral and normal tissues (data not shown). Interestingly, clinicopathological analysis indicated that regional LNM, including lateral neck node metastasis (N1b), was more frequent in PTCs that exhibited no change in expression of MRPL44 mRNA ($P=0.001$, Table 1). Moreover, multivariate analysis clearly indicated that no change in MRPL44 expression (indicated as Group 2) increased the risk of lateral neck LNM after adjustment for age, ETE, T-stage, and multifocality (odds ratio 9.267, 95% confidence interval 1.852–46.371, $P=0.007$, Table 2).

**DISCUSSION**

Otto Warburg$^{11}$ discovered that most cancer cells produce cellular ATP using a high rate of glycolysis instead of OxPhos. This observation can be clinically detected by performing uptake imaging using FDG PET as shown in Figure 4B. The Warburg effect might be related to mitochondrial damage in cancer cells, or to adaptation to hypoxia within the tumor microenvironment. Recently, the aberrant expression of glycolytic enzymes, such as overexpression of mitochondrial bound hexokinase and the M2 splice isoform of pyruvate kinase, was reported as a possible cause of the Warburg effect. However, because mitochondria can produce much more cellular ATP than via glycolysis, cancer cells still use mitochondria to produce ATP for fatty acid synthesis and other biosynthetic processes. In agreement with these recent observations concerning mitochondrial metabolism in cancer cells, the GSEA data showed that OxPhos and MR gene sets are coordinately downregulated in most PTCs, indicating that PTC cells rely primarily on aerobic glycolysis for their energy needs. However, 16.3% of PTCs in the GSE33630 data set and 15.5% of PTCs in our cohort had the same amount of OxPhos and MRP mRNA expression as normal thyroid tissues. In line with our data, HPA also indicated that MRPL44 and NDUFA5 gapdh mRNAs were downregulated in PTCs compared with normal thyroid tissues.
TABLE 2. Multivariate Analysis of the Association of LNM (N1b) With Levels of Expression of MRPL44

| MRPL44 mRNA Expression | Decreased | No Change | P Value |
|------------------------|-----------|-----------|---------|
| Age, y                 | N = 87, n (%) | N = 16, n (%) |         |
| 46.4 ± 1.437           | 47.56 ± 4.148 | 0.76*      |
| Tumor size, cm         | 2.209 ± 0.098 | 2.319 ± 0.183 | 0.65*
| T-stage                |           |           |         |
| T1a                    | 7 (8.0) | 0 | 0.22
| T1b                    | 15 (17.2) | 4 (25.0) |   |
| T2                     | 21 (24.1) | 0 |   |
| T3                     | 44 (50.6) | 12 (75.0) |   |
| T4                     | 0 | 0 |   |
| ETE                    |           |           |         |
| Negative               | 45 (51.7) | 4 (25.0) | 0.061
| Minimal                | 42 (48.3) | 12 (75.0) |   |
| Extensive              | 0 | 0 |   |
| Multifocality          |           |           |         |
| Negative               | 70 (80.5) | 12 (75.0) | 0.721
| Unilateral             | 7 (8.0) | 1 (6.3) |   |
| Bilateral              | 10 (11.5) | 3 (18.8) |   |
| Regional lymph node    |           |           |         |
| N0                     | 40 (46.0) | 5 (31.3) | 0.001
| N1a                    | 42 (48.3) | 5 (31.3) |   |
| N1b                    | 5 (5.7) | 6 (37.5) |   |
| Distant metastasis     |           |           |         |
| M0                     | 87 (100) | 15 (93.8) | 0.16
| M1                     | 0 | 1 (6.3) |   |
| TNM stage group        |           |           |         |
| I                      | 43 (49.4) | 7 (43.8) | 0.14
| II                     | 10 (11.5) | 1 (6.3) |   |
| III                    | 6 (6.9) | 0 |   |
| IVA                    | 25 (28.7) | 5 (31.3) |   |
| IVB                    | 3 (3.4) | 3 (18.8) |   |
| IVC                    | 0 | 0 |   |

ETE = extrathyroidal extension, mRNA = ??, MRPL44 = mitochondrial ribosomal protein L44, PTC = papillary thyroid cancer, TNM = ??.

*P values calculated by unpaired t test. Data are mean ± SD.

1 P values calculated by χ² test or linear-by-linear association.

In conclusion, our data suggest that a proportion of PTCs have a combined (oxidative and glycolytic) metabolic phenotype, even though they still rely on glycolytic activity for ATP generation. In addition, the metabolic phenotype can be determined by measuring the expression of MRPL44, which we show to be a promising marker for the prediction of lateral neck LNM. Future studies should focus on the metabolic phenotyping of PTCs according to their level of MRPL44 expression.
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