Research article

High RPMB predicts poor disease-free survival of male N1 papillary thyroid cancer after adjuvant radioiodine therapy

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HIGHLIGHTS

- We established a new biomarker named RPMB in an attempt to fulfill this mission.
- A high RPMB was significantly associated with poor disease-free survival (DFS) in male PTC patients.
- Kaplan-Meier analysis showed that high RPMBs could significantly predict poor DFS in male patients after R0 resection for N1 disease.
- Both male sex and high RPMB were proven as independent unfavorable factors for DFS after adjuvant RAI therapy.
- RPMB might be a potential predictor to identify suitable male PTC patients who can benefit from adjuvant RAI therapy.

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ABSTRACT

The current recommendation for the use of adjuvant radioactive iodine (RAI) therapy in papillary thyroid cancer (PTC) after radical surgery is based on clinicopathological factors; however, this recommendation remains controversial. Our present study established a new biomarker, RPMB (promotor methylation burden of DNA repair genes (DRGs)), to identify a patient subgroup suitable for adjuvant RAI therapy. We defined RPMB as the ratio of methylated DRGs to the total number of DRGs. Methylation profiles of 498 PTC tumors and their clinical data were retrieved from the Cancer Genome Atlas (TCGA) database. DRGs of PTC subjects were found to be much more hypomethylated than controls across the whole profile (all p < 0.001). PTC patients with higher RPMBs tended to be >45 years old and female, and these PTCs were commonly unifocal, with N0 disease, wild-type BRAF, and mutated RAS. The subgroup analysis indicated that high RPMBs were significantly associated with poor disease-free survival (DFS) in male patients with PTC (HR = 4.855, 95% CI: 1.527–15.433, p = 0.007). Moreover, Kaplan-Meier analysis showed that high RPMBs could significantly predict poor DFS in male patients after R0 resection for N1 disease (HR: 5.431, 95% CI: 1.045–28.219, p = 0.024), and the p-value was very close to significance in these patients after adjuvant RAI therapy (HR: 6.269, 95% CI: 0.693–56.714, p = 0.062). Multivariate analysis indicated that both male sex (HR: 14.565, 95% CI: 2.153–98.507, p = 0.006) and high RPMBs (HR = 11.206, 95% CI: 1.622–77.405, p = 0.014) were independent unfavorable factors for DFS after adjuvant RAI therapy. Therefore, RPMB might be a potential predictor for identifying suitable male patients with PTC who can benefit from adjuvant RAI therapy.

1. Introduction

Differentiated thyroid carcinomas (DTC) have good clinical outcomes, with 10-year overall survival (OS) rates exceeding 90% according to the AJCC Cancer Staging Manual, 8th edition. DTC, including papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and Hurtle-cell thyroid cancer (HTC), accounts for more than 90% of all thyroid cancer cases, among which PTC accounts for up to 80% of diagnosed cases [1]. The annual number of new cases nearly tripled from 1975 (4.9/100,000) to 2009 (14.3/100,000), and the increased morbidity and mortality of PTC is considered responsible for most of this change [2]. Up to 30% of DTC patients will experience tumor recurrence after initial therapy in the following decades, of which 66% occur within the first decade [3].
Despite the much higher morbidity of PTC in women, it is putatively accepted that women have a more favorable prognosis than men. OS was reported to be significantly improved in women in several retrospective studies [4]. Male PTC patients were also reported to have worse disease-free survival (DFS) after initial therapy compared to female patients [5]. Meanwhile, PTCs in male patients also manifest more aggressive clinical features than in women, including larger tumor size, lymph node metastases, extrathyroidal extension, and distant metastases [6].

Among the numerous staging systems for PTC, according to the worldwide literature [7], the American Thyroid Association (ATA) risk stratification system is the most popular. It utilizes clinicopathologic factors to categorize PTC patients into low-, intermediate-, or high-risk groups according to the patients’ possibility of disease recurrence. Currently, the evidence for ¹³¹I adjuvant radioactive iodine (RAI) therapy after radical surgery is relatively weak. Considering that the prognosis of PTC after initial therapy is very good, no prospective randomized clinical trials (RCTs) have been conducted to specify the certain prognostic indicators (clinicopathological features or molecular biomarkers) that guide the decision making of adjuvant RAI therapy. Notably, conclusions from retrospective studies are often controversial.

To cope with the global heterogeneity in PTC clinical implementations, the ATA recommended the application of adjuvant RAI therapy for PTC based on ATA risk stratifications according to clinicopathologic features. Generally, the evidence for adjuvant RAI therapy among ATA low- and intermediate-risk stratifications is either equivocal or inconclusive [8, 9]. Nevertheless, a prospective non-randomized study showed a significant benefit in DFS, OS, and disease-specific survival (DSS) in stage III/IV patients who received adjuvant RAI therapy [10]. Thus, despite the lack of strong evidence from prospective RCTs, routine adjuvant RAI treatment is recommended for ATA low-risk patients. However, as in any other categorical staging system, a particular patient might be evaluated as belonging to different risk categories depending on their clinical features. Furthermore, the evidence of the ATA risk stratification system contains a large amount of conflicting or non-conclusive data, especially for low- and intermediate-risk groups [7]. In addition, the 2015 ATA risk stratification system introduced some new hazardous factors, such as multifocality, genotype, vascular invasion, or metastatic lymph nodes [11, 12], which were ignored by the 2009 ATA system. However, it is still unclear as to how these factors affect recurrence risk quantitatively, how they interact with others, and whether other variables should be included, making decision making in further management very difficult [7].

The role of molecular biomarkers in guiding adjuvant RAI therapy has yet to be established. The most widely studied molecular biomarker is BRAF V600E. Although preclinical studies have indicated that the BRAF V600E mutation could significantly reduce the expression of sodium-iodide symporter (NIS) and thereby affect radioidine uptake [13], the evidence for BRAF V600E as a prognostic indicator for adjuvant RAI is far from sufficient. One subgroup analysis from Italy reported a recurrence difference between wild-type BRAF and BRAF V600E-mutant PTC patients with early stage disease (T1aN0M0). In the aforementioned study, 97 patients received postoperative RAI therapy, whereas the rest did not. DFS was significantly lower in BRAF V600E-mutant patients than in wild-type patients who received adjuvant RAI therapy [14]. There were some issues preventing the meaningful analysis of the relationship between the BRAF V600E mutation and adjuvant RAI therapeutic efficacy, such as the relatively small number of patients who did not undergo adjuvant RAI therapy, the small proportion of disease recurrences in these patients, and the lack of randomization in these studies. Despite the uncertainty regarding the BRAF V600E mutation, novel molecular biomarkers for guiding adjuvant RAI therapy are greatly needed, as is intensive research in this area.

DNA methylation has been broadly reported to be essential for promoting embryo development [15], aging [16], and carcinogenesis [17] by re-arranging the structures of normal DNA and chromatin [18]. Promoter dysregulation has been proven to play a very important role in cancer transformation and biomarker discovery [19].

The idea of using RPMB as an indicator for PTC adjuvant RAI therapy was inspired by the close interplay between DNA repair and genomic instability. Defects in DNA damage repair systems are a pervasive hallmark of cancer cells, leading to large-scale genomic instability. The molecular events that affect the process of DNA damage repair provide opportunities for biomarker discovery and therapeutic intervention [20]. Genomic instability present in tumor cells can act as a driving force to promote carcinogenesis by gradually increasing the rate of spontaneous mutations, causing aggressive biological behaviors in tumor cells [21]. Hence, we hypothesized that promoter methylation of DNA repair genes (DRGs) might enhance the aggressiveness of PTC tumor cells by virtue of genomic instability, thereby counteracting the efficacy of RAI therapy. Our results demonstrated that RPMB was a prognostic factor for predicting DFS in male PTC patients with lymph node metastasis after radical surgery, and the predictive power remained for the subgroup of patients who had received adjuvant RAI therapy.

The Cancer Genome Atlas (TCGA) database is an extremely valuable resource of genetic and epigenetic information that provides publicly available datasets for a variety of cancer types [22]. Although clinical data are severely scarce, we could still collect the data of male PTC patients who received both radical resection and adjuvant RAI therapy. We also aimed to establish a promising predictor, named RPMB, short for promoter methylation burden of DRGs, in order to find proper PTC patients suitable for adjuvant RAI therapy. The association between DNA repair and radiotherapy efficacy has been widely reported, especially in gliomas [23]. We hypothesized that RPMB, a measure of the methylation level of DRGs, is likely closely related to the efficacy of adjuvant RAI therapy for PTC, and thereby has the predictive ability to identify suitable PTC patients who can benefit from this treatment.

2. Materials and methods

2.1. Data collection

The methylation profiles of PTCs and their clinical data were retrieved from Bioconductor R package “RTCGA” and “TCGAbiolinks” in October 2020. The PTC clinical dataset contained the clinicopathological information of 507 patients with PTC, of which many values were missing. The methylation profiles included 498 PTC tumors. The methylation values of each CpG site were presented as β-values ranging from 0 to 1 (from unmethylated to fully methylated). The promoter genomic region was defined as in our previous study, ranging from 1,000 bp upstream to 300 bp downstream of the transcription start site [24]. The mean β-values of the CpG sites that were mapped onto the promoter regions of particular genes were used as the promoter methylation values of these genes. Thus, the methylation profiles of 20,102 genes were eventually determined.

The clinicopathological characteristics of the PTC patients were collected, including sex, age, ethnicity, laterality (left lobe, right lobe, or bilateral/isthmus), histology (classic, follicular, or tall cell), tumor foci (unifocal or multifocal), pathological lymph node (pN), pathologic tumor size (pT), BRAF V600E mutation, and RAS (including KRAS, NRAS, and HRAS) mutations. We first divided the PTC patients into low- and high-RPMB groups based on the median value. The chi-square test was used to analyze the association between RPMB and clinicopathological characteristics.

2.2. Collection of DRGs and comparison of their methylation levels with other genes

The gene list of the DNA repair genes was obtained from the Gene Ontology (GO, http://www.geneontology.org) database using the GO term "GO:0006281." We found 552 DRGs using this GO term, and 530 could be found in the TCGA PTC data. First, the methylation values of 530 DRGs were
Figure 1. Schematic diagram of this study. The TCGA PTC clinical dataset contained the clinicopathological information of 507 patients. A total of 442 patients had DFS data, among whom 83 were male patients that received radical surgery. The methylation profile included 498 PTC tumors, and RPMB was calculated for each PTC patient. The RPMB values of 30 male patients who had received adjuvant RAI therapy after radical surgery were eventually collected.

Figure 2. Boxplots of the promoter methylation values of the DRGs and other genes. A. Boxplot of the methylation levels of the DRGs and the other 10 sets of randomly sampled genes. B. Boxplot of the methylation levels of the DRGs and those within the other 10 GO terms.
compared with those of 530 non-DRGs, which were randomly selected across the genome 1,000 times. Next, the unpaired t-test was used to compare the methylation values of the 530 DRGs with those randomly selected gene set. Furthermore, the methylation values of the DRGs were compared with those among the 10 other GO terms based on unpaired t-tests, as these genes have essential functions during the process of cancer transformation, including cell proliferation (GO:0008283), cell death (GO:0008219), secretion (GO:0046903), apoptotic process (GO:0006915), cell migration (GO:0016477), cell development (GO:0048468), angiogenesis (GO:0001525), morphogenesis (GO:0000902), immune response (GO:0006955), and cell adhesion (GO:0007155).

2.3. Calculation of RPMB values

A cut-off β-value of 0.2 was used to categorize the 530 DRGs into two groups of genes: “methylated” (β-value > 0.2) and “unmethylated” (β-value ≤ 0.2). Therefore, the RPMB of a particular patient was determined as the ratio of methylated DRGs to all DRGs (n = 530).

2.4. Correlation of RPMB with DFS

Cox regression analysis was evaluated to use the treatment effects of RPMB on PTC patients’ DFS within each level of the clinical variables separately, and we displayed the corresponding results in the form of a forest plot. We divided the PTC patients into low- and high-RPMB groups according to the median value, and Cox regression analysis was performed to quantify the association between RPMBs and different clinical characteristics using 95% confidence intervals (CIs) and hazard ratios (HRs). Eventually, 442 PTCs were collected based on the following criteria: i. no neoadjuvant therapies ever received; ii. patients undergoing radical surgery and regional lymph node dissection; and iii. DFS data available for further analysis.

2.5. Statistical analysis

Statistical analyses in this study were carried out using R programming software (Version 3.6.0) and Bioconductor R packages (Version 3.9). The gene lists of different GO terms were downloaded from the annotation package “org.Hs.sg.db” (version 2.8.0) [25]. Kaplan–Meier survival analysis and log-rank test were used to show the survival differences between the different RPMB groups, which were divided according to the median RPMB value. Univariate and multivariate Cox analyses with RPMB and other clinicopathological factors were conducted to identify independent prognostic factors of PTCs after adjuvant RAI therapy.

3. Results

The schematic diagram is illustrated in Figure 1.

3.1. Comparison of RPMB (low/high) between DRG genes and other groups of genes (randomly or biologically selected)

First, the methylation values of 530 DRGs were compared to those of other randomly chosen non-DRGs. The results showed that the median methylation value of the DRGs was 0.170, and this number was much lower than those for the other 1,000 non-DRG sets (Figure 2A shows the boxplot of the 10 selected gene sets, all p < 0.001). Moreover, we collected 10 sets of genes within other GO terms, and the methylation values of these genes were compared to those of the DRGs. The ten GO terms were closely related to important biological characteristics of cancers, including angiogenesis, immune response, apoptotic process, cell death, cell migration, morphogenesis, cell development, secretion, cell adhesion, and cell proliferation. Surprisingly, the methylation values of the DRGs were also found to be much lower than those of any other gene sets that were related to other cancer-related biological processes (Figure 2B, all p < 0.001).

3.2. Correlation of RPMB (low/high) with clinical-genetic features in the overall population

The association between RPMB and the clinicopathological characteristics of the PTC patients is shown in Table 1. PTC patients were divided into low- and high-RPMB groups based on the median value. The chi-square test indicated that RPMB was significantly related to sex, focality, pN status, BRAF V600E, and RAS mutations (Table 1). PTCs with higher RPMBs were mostly present in female patients (Wald χ² = 106.290, p = 2.2 × 10⁻¹⁶), unifocal (Wald χ² = 5.251, p = 0.022), pN0 (Wald χ² = 7.699, p = 0.006), with wild-type BRAF (Wald χ² = 6.456, p = 0.011), and involved mutated RAS (Wald χ² = 6.078, p = 0.014). Other clinical variables, such as age, ethnicity, laterality, histology, and tumor size, were well balanced between the two RPMB patient groups.

3.3. Correlation of RPMB (low/high) with DFS of the overall study population

We further evaluated the treatment effects of RPMB on PTC patients’ DFS within each level of the clinical variables separately, and displayed the corresponding results in the form of a forest plot (Figure 3). Eventually, 442 PTCs were collected based on the criteria mentioned in the Methods section. The forest plot showed that the hazard ratio for DFS in all patients was 1.111 (95% CI: 0.623–1.982, p = 0.721). None of the subgroup analyses were significant, except that male patients with PTC tended to have low RPMBs (HR = 4.855, 95% CI: 1.527–15.433, p = 0.007). Non-

Table 1. Association between RPMB and the clinical characteristics.

| Characteristics | Low RPMB | High RPMB | χ² | p  |
|-----------------|----------|-----------|-----|----|
| Age (years)     |          |           |     |    |
| <45             | 126      | 98        | 1.906 | 0.167 |
| ≥45             | 136      | 138       |      |     |
| Sex             |          |           |     |    |
| Male            | 120      | 11        | 106.290 | <2.2 × 10⁻¹⁶ |
| Female          | 142      | 225       |      |     |
| Ethnicity       |          |           |     |    |
| Asian           | 21       | 30        | 2.867 | 0.239 |
| White           | 171      | 157       |      |     |
| Black or others | 16       | 11        |      |     |
| Location        |          |           |     |    |
| Left lobe       | 91       | 85        | 3.059 | 0.217 |
| Right lobe      | 107      | 105       |      |     |
| Bilateral/isthmus | 63   | 41        |      |     |
| Histology       |          |           |     |    |
| PTC-classic     | 199      | 160       | 4.330 | 0.115 |
| PTC-follicular  | 45       | 57        |      |     |
| PTC-tall cell   | 18       | 19        |      |     |
| Tumor focality  |          |           |     |    |
| Unifocal        | 127      | 139       | 5.251 | 0.022 |
| Multifocal      | 130      | 92        |      |     |
| pN status       |          |           |     |    |
| N⁻              | 107      | 121       | 7.699 | 0.006 |
| N⁺              | 133      | 87        |      |     |
| pT status       |          |           |     |    |
| T1/T2           | 157      | 152       | 0.895 | 0.344 |
| T3/T4           | 104      | 83        |      |     |
| BRAF V600E      |          |           |     |    |
| Wild type       | 71       | 92        | 6.456 | 0.011 |
| Mutation        | 134      | 101       |      |     |
| RAS             |          |           |     |    |
| Wild type       | 187      | 159       | 6.078 | 0.014 |
| Mutation        | 18       | 34        |      |     |
significant subgroups with HRs \( >1.2 \) or \( <0.8 \) were regarded as marginally correlated. The subgroups marginally favoring low RPMBs included younger patients (HR \( = 1.306, 95\% \text{ CI: } 0.543–3.140, \ p = 0.55 \)), PTCs located at bilateral or isthmus (HR \( = 1.609, 95\% \text{ CI: } 0.465–5.562, \ p = 0.453 \)), unifocal disease focality (HR \( = 1.698, 95\% \text{ CI: } 0.750–3.844, \ p = 0.204 \)), tumor sizes \( \geq T3 \) (HR \( = 1.264, 95\% \text{ CI: } 0.602–2.655, \ p = 0.537 \)), pN0 disease (HR \( = 1.921, 95\% \text{ CI: } 0.656–5.622, \ p = 0.333 \)), R1/2 resection (HR \( = 1.773, 95\% \text{ CI: } 0.440–7.150, \ p = 0.421 \)), wild-type BRAF (HR \( = 2.298, 95\% \text{ CI: } 0.463–11.040, \ p = 0.309 \)), and mutated RAS (HR \( = 1.828, 95\% \text{ CI: } 0.201–16.639, \ p = 0.593 \)). The only subgroup that marginally favored high RPMB was multifocal disease (HR \( = 0.700, 95\% \text{ CI: } 0.281–1.741, \ p = 0.443 \)).

### 3.4. Correlation of RPMB (low/high) with DFS in PTC patients subjected to RAI therapy

There were many missing values within the TCGA clinical data, particularly in terms of treatment-related parameters. Thus, only 112 male patients that received radical resection were included in the detailed DFS analysis. Additionally, 83 male patients were confirmed to have undergone R0 resection, among whom 30 patients were documented to have received adjuvant RAI therapy.

RPMB values were significantly different between male and female patients (Figure S1, \( t \) statistic \( = -17.393, \ p < 2.2 \times 10^{-16} \)). The RPMB for male patients ranged between 0.130 and 0.192, with a mean value of 0.15. Therefore, in the following DFS analysis, 0.15 was adopted as the cutoff value with which to categorize PTC patients into low or high RPMB subgroups (Figures 4 and 5). Survival analysis of DFS was conducted in all male patients after radical surgery (Figure 4A), male patients after R0 resection (Figure 4B), lymph node-positive male patients after R0 resection (Figure 4C), and patients in the aforementioned three groups who also underwent adjuvant RAI therapy (Figure 4D–F). The results indicated that the tendency for poor DFS in male PTC patients with high RPMB was consistent in all subgroup analyses. High RPMB was significantly associated with poor DFS in male patients after R0 resection for N1 disease (Figure 4C; HR, 5.431; 95\% CI, 1.045–28.219; \( p = 0.024 \)). The number of male patients after adjuvant RAI therapy was relatively limited due to missing values, with a total of 39 male patients after...
adjuvant RAI therapy (Figure 4D), 30 patients who received both R0 resection and adjuvant RAI therapy (Figure 4E), and 17 patients with additional positive lymph nodes (Figure 4F) included. Although the number of patients decreased due to added-in constraints, surprisingly, the log-rank \( p \)-value continued to decrease. Figure 4F shows that the \( p \)-value was very close to significance in 17 patients (HR: 6.269, 95% CI: 0.693–56.714, \( p = 0.062 \)), implying that RPMB might be a promising indicator for predicting the DFS of lymph node-positive male patients after R0 resection and adjuvant RAI therapy.

We also re-evaluated the prognostic power of other putatively accepted clinical factors, such as BRAF V600E mutation, tumor size (T1/2 vs. T3/4), and positive lymph node ratio (>0.5% vs. <0.5%), in pN1 male patients after R0 resection (Figure 5A–C) and in the three aforementioned groups of patients after adjuvant RAI (Figure 5D–F). None of these factors were significantly associated with DFS. Tumor size presented the best performance among all three factors (pN1 male patients after R0 resection: HR = 2.933, 95% CI = 0.565–15.211, \( p = 0.18 \); Figure 5B; pN1 male patients after R0 resection and adjuvant RAI therapy: HR = 2.69 \times 10^0, \( p = 0.23 \), Figure 5E).

3.5. Adjustment of clinicopathological features impacting prognosis (multivariate analysis)

Table 2 shows the univariate and multivariate Cox analyses of RPMB with other clinicopathological factors, such as age, sex, pathologic N, and extrathyroidal invasion in PTCs subjected to adjuvant RAI therapy. Eventually, the data of 99 patients with PTC were collected using the aforementioned variables. Although the univariate analysis did not show any significant associations, the results of the multivariate analysis indicated that both male sex (HR = 14.565, 95%CI: 2.153–98.507, \( p = 0.006 \)) and high RPMBs (HR = 11.206, 95%CI: 1.622–77.405, \( p = 0.014 \)) were independent unfavorable factors for DFS after adjuvant RAI therapy (Table 2).

4. Discussion

Several staging systems have been established to predict the prognosis of patients with PTC [26]. All of these systems use a combination of clinical factors such as age, tumor size, histology, extrathyroidal invasion, and distant metastasis to stratify patients into different categories with different prognoses. For example, a nomogram was developed using the SEER database [27], the MACIS system from the Mayo Clinic [28], and the simplified TNM system using a quantitative approach similar to the MACIS system [29]. However, none of these staging systems showed clear superiority when compared with the others, and none of the staging systems could predict the mortality of PTC patients with satisfactory performance [30]. This inaccuracy in predicting the prognosis of an individual PTC patient may be due to the low quality of clinical evidence and failure to adequately and precisely integrate the risk estimation based on potentially important clinicopathological factors. For instance, some studies questioned the cutoff of 45 years that was being used to upstage PTC patients based on retrospective studies, and this cutoff has been modified to 55 years old in the 8th edition of the AJCC staging system [31]. Therefore, the current risk stratifications based on clinicopathologic factors are not consistently reliable, and there is still no molecular biomarker with strong predictive power for risk stratification, let alone for decision making in adjuvant RAI therapy.

The BRAF V600E mutation is regarded as the most promising molecular biomarker to fulfill the aforementioned mission. However, some studies have reported that although the presence of the BRAF V600E
mutation is identified in approximately two-thirds of PTCs with lymph node metastases, BRAF status alone shows a very limited predictive ability to detect PTCs with hazardous clinical features [32, 33]. Hence, a new molecular biomarker with predictive value in disease recurrence and clinical decision making is greatly needed, in addition to traditional clinicopathologic factors.

The prognosis of PTC is less favorable in men than in women, and one study showed that the mortality rate of PTC was double in men compared to women [3]. Therefore, male PTC patients should be treated with more aggressive clinical interventions, especially those aged ≥40 years [34]. ATA risk stratification does not take sex differences into consideration, whereas the adoption of adjuvant RAI therapy for the different sexes must be tailored carefully due to its prognostic disparity. The RPMBs between male and female patients with PTC were significantly different from each other (Figure S1), suggesting that RPMB plays a very important role in the sex disparity of PTC prognosis. A novel molecular indicator is essential to help researchers identify PTC patients who are suitable for adjuvant RAI therapy, especially male patients, as the underlying molecular mechanisms in the two sexes must be quite distinct. In this study, we aimed to discover a new molecular indicator to screen for suitable patients who can benefit from adjuvant RAI therapy and ideally spare those insensitive to this treatment from unnecessary exposure to radiation.

The association between methylation of DRGs and radiotherapy is also well known in malignant glioma, in which MGMT methylation was reported as a prognostic biomarker that can predict potential benefits from chemotherapy medications such as nitrosoureas [35] and temozolomide [36]. In addition, MGMT hypermethylation has been proven in two RCTs to improve the prognosis of glioma patients who received both radiotherapy and temozolomide [37]. Furthermore, we first reported the predictive ability of RPMB in adjuvant radiotherapy for gastric cancer, in which high RPMB was closely related to better DFS after adjuvant radiotherapy [38]. However, the radiobiological effect of RAI in PTC is quite different from that of radiotherapy with regular fractionation in glioma or gastric cancer. The biological theory of radiotherapy involves vital macromolecules that target, either directly or indirectly, the DNA biostructure. Inactivation of DRGs by promoter hypermethylation can intensify the DNA-damaging bioeffect caused by radiotherapy with regular fractionation, leading to a more favorable clinical outcome. However, under most circumstances, adjuvant RAI therapy in PTC is applied using a single administration of 131I radiiodine, the dosage of which is highly concentrated in the remnant thyroid tissues and tumor cells. Thus, the lethal damage induced by adjuvant RAI therapy cannot be repaired or reversed by DNA repair mechanisms. The distinct radio-biological effects of RAI and the regular fractionation of external beam radiation might explain why high RPMB (contributing to more aggressive cellular behaviors) predicted unfavorable DFS in male PTC patients, whereas the opposite was true (reduced repairing ability of tumor cells after radiotherapy with regular fractionation) in glioma and gastric cancer.

The methylation patterns of the PTCs and gastric cancers in our previous study were highly consistent. First, the promoter region of the DRGs was greatly hypomethylated compared to the others, and the same phenomenon was also observed in gastric cancer [38]. Thus, it can be inferred that hypomethylation of DRGs could activate the DRGs as a protective maneuver attempting to redirect tumor cells onto the normal bio-behavioral pathway. Although PTC affects women more frequently, men display more aggressive clinical and histological features. The methylation values of DRGs were much higher in women in both cancer

Figure 5. DFS analysis with different known hazardous factors in male N1 PTC patients after R0 and those after adjuvant RAI therapy. A. DFS analysis between wide-type BRAF and BRAF V600E mutant PTC patients. B. DFS analysis between T1/2 and T3/4 PTC patients. C. DFS analysis between PTC patients with lymph node positive ratios ≥0.5 and those with ratios <0.5. D. DFS analysis of the PTC patients in Figure 5A after adjuvant RAI therapy. E. DFS analysis of the PTC patients in Figure 5B after adjuvant RAI therapy. F. DFS analysis of the PTC patients in Figure 5C after adjuvant RAI therapy.
Table 2. Univariate and multivariate analyses of DFS after adjuvant RAI therapy.

| Factors | Univariate Cox regression | Multivariate Cox regression |
|---------|---------------------------|-----------------------------|
|         | HR (95% CI) | P     | HR (95% CI) | P  |
| Age (>45/<45) | 1.560 (0.495–4.918) | 0.448 | 0.793 (0.234–2.693) | 0.710 |
| Sex (Male/Female) | 2.884 (0.929–8.952) | 0.067 | 14.565 (2.153–98.507) | 0.006 |
| Extra-thyroid invasion (yes/no) | 3.057 (0.920–10.153) | 0.068 | 2.444 (0.668–8.939) | 0.177 |
| Pathologic N (N1/N0) | 3.585 (0.784–16.391) | 0.100 | 4.103 (0.791–21.281) | 0.093 |
| RPMB (high/low) | 1.729 (0.548–5.456) | 0.350 | 11.206 (1.622–77.405) | 0.014 |

Abbreviations: HR, hazard ratio; CI, confidence interval. Note: Significant p-values are shown in bold (p < 0.05).

types, implying that RPMB might be responsible for the sex disparity in the clinical outcomes. The consistent RPMB patterns in these two cancers are certainly not a coincidence, and further research on RPMB in other cancer types is needed to depict the pattern of DRG methylation from a more extensive perspective.

Table 1 shows the association between RPMB and the clinical characteristics, regardless of patient prognosis. The results in Table 1 indicate that high RPMB is associated with some favorable factors, such as female sex, pN0, and wild-type BRAF, while high RPMB seemed to be a risk factor for recurrence of PTC after adjuvant RAI therapy. RPMB was high in the subgroup of patients with good prognoses; thus, RPMB must be a favorable prognostic predictor in other patients. However, this might not be true, as the two subgroups of patients were very different in many respects. Carcinogenesis is a complicated biological process that involves a variety of signaling pathways, such as cell proliferation, inflammation, and vascularization. Promoter methylation of DRGs is surely one of them. Women might be biologically immune to PTC carcinogenesis, possibly due to advantages in the other signaling pathways, and thus, the expression of DNA repair proteins is not urgent. Therefore, women possess high RPMB, while low RPMB might be a genetic maneuver to protect men from thyroid carcinogenesis. Another possible explanation for this contradiction might be stereotypic thinking described in our previous study [39].

A routine pipeline seems very common in many cancer studies, and expression differentiation may be helpful in identifying prognostic molecules. There also exists a striking unanimity that molecules upregulated in cancer usually shorten survival, whereas downregulated molecules have the opposite effect. Contrary to our stereotypical research pattern, expression differentiation between cancer and adjacent normal tissues was proven to be irrelevant to the corresponding survival correlation, and the directions of the dysregulated genes in PTC were irrelevant to the direction of the corresponding survival correlation. Therefore, the direction of dysregulation cannot predict patient prognosis.

Due to the many missing values in the TCGA clinical dataset, the limited sample size in this study is one of the limitations. For example, only 43 N1 male patients with PTC undergoing R0 resection were available for DFS analysis, and only 17 patients among these received adjuvant RAI therapy. Thus, increasing the number of patients in this study is important for strengthening our conclusions. Moreover, to the best of our knowledge, the TCGA PTC dataset is the only dataset that contains both methylation profiles and clinical outcomes after adjuvant RAI therapy in patients with PTC. Thus, it is currently impossible to consolidate the predictive power of RPMB in another independent cohort, confirm a proper β-value as a cutoff to binarize methylated or unmethylated DRGs, and generate an RPB threshold to help us find PTCs suitable for adjuvant RAI therapy. In the future, a prospective study will be carried out to further demonstrate our findings on RPMB’s potential clinical usage in PTC, determine the two aforementioned cutoffs, and downsize the current 530 DRGs to a more manageable gene set to increase the feasibility of RPMB testing in clinical practice.

5. Conclusions

The findings of this study demonstrated that RPMB might be considered a promising new biomarker for identifying PTC patients suitable for adjuvant RAI therapy.

Declarations

Author contribution statement

Ning An: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xue Yang: Conceived and designed the experiments; Performed the experiments; Contributed materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at Thyroid carcinoma (THCA) data in The Cancer Genome Atlas (TCGA) database.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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