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# The reverse molecular dysregulations caused by BRAF and NRAS mutations in papillary thyroid cancer

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**Abstract:** Background BRAF and NRAS mutations are two major somatic mutations extensively investigated in papillary thyroid cancer (PTC). However, much fewer studies have been conducted to investigate the underlying molecular mechanic difference caused by the two mutations. Methods Multi-level genomic data of 496 PTC samples and corresponding clinical data were all retrieved from TCGA database. Clinico-pathological factors and molecular dysregulations were compared between PTC samples with different BRAF and NRAS mutational status. Results BRAF and NRAS mutations were both significantly associated with histological diagnosis, extrathyroidal invasion and lymph node metastasis in PTC(all $p < 0.01$), and BRAF mutation might promote invasion and migration, while NRAS mutation works the otherwise. Neither DNA copy numbers(FDR < 0.01, fold change > 1.5) nor methylations(FDR < 0.01, $|\Delta| > 0.2$) were significantly altered by these mutations, while mRNA dysregulations were commonly observed. Additionally, mRNAs differentially expressed in both BRAF and NRAS mutations tended to be dysregulated at reverse directions. T cell regulation was the major biological function of these reversely dysregulated mRNAs. Furthermore, Fibronectin was over-expressed in BRAF mutated while under-expressed in NRAS mutated patients($p=6.492\times10\text{-}7$). Conclusions The mRNA expression patterns were significantly reversed between BRAF and NRAS mutated PTC samples. These oppositely dysregulated mRNAs were significantly related to T lymph cell regulation and immune response.

**Keywords:** Papillary thyroid cancer; Somatic mutation; BRAF; NRAS; Clinico-pathological association.

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## 1. Introduction

Papillary thyroid cancer (PTC) accounts for nearly 80% of all thyroid cancer cases[1,2]. Although the overall outcome is excellent and its 10-year survival rate is approximately 90%[3,4], lymph node metastasis and distant metastasis are still the major causes of PTC mortality[5,6]. Approximately 50% of PTC patients show lymph node metastasis and approximate proportion of distant metastasis is around 5-7%, in most circumstances, involving lungs and bones[7]. In the last two decades, the researches upon somatic mutations of essential pathways critical to PTC carcinogenesis has dramatically increase our understanding of the intricate underlying molecular mechanisms about this malignancy[8-10]. It is reported that oncogenic mutation has been detected in 97% of PTC patients[11], among which BRAF and NRAS mutations are the most extensively investigated. BRAF is the most commonly mutated gene, accounting for nearly 60% of PTC patients, and BRAF mutation can persistently activate Serine/Threonine kinase through MAPK/ERK signaling pathway[12]. A variety of studies demonstrated that BRAF mutation was closely related to many unfavorable clinico-pathological factors, including lymph node and extrathyroidal invasion, leading to the increased risk of aggressiveness and recurrence, as well as a poor prognosis of PTC patients[13-15]. Additionally, the immune-histological and cytological studies reported that BRAF mutation could render sodium iodide symporter (NIS) silenced and downregulate its expression in PTC cells[16,17], and the following clinical studies also indicated a strong association between BRAF mutation and the radioiodine resistance of distantly metastatic foci[18]. However, there are still conflicting perspectives with regard to the relationship between BRAF mutation and PTC's aggressiveness, for instances, extrathyroidal extension and lymph node metastasis[19,20]. Barabaro et al. also reported no significant association between BRAF mutation and lymph node metastasis with cytology smears[21].

NRAS mutation is commonly found in many human cancers[22]. RAS family gene mutation is the second most common mutation detected in fine needle aspiration (FNA) samples from thyroids[23,24], among which NRAS mutation is the most prevalent[25,26]. RAS mutations might activate the PI3K/AKT pathway in the process of thyroid carcinogenesis, since the preferential association of RAS mutations with AKT phosphorylation was observed in thyroid cancers[27,28]. Although the prevalence of NRAS mutation in PTC, its current studies remain focusing on its mutation in follicular variant of PTC[20,29], with the corresponding mutation rate ranging from 0% to 23% in classical histological type, and from 25% to 43% in follicular

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variant of PTCs[30-34]. RAS mutations were reported to be the predominant molecular abnormalities in poorly differentiated thyroid carcinomas and associated with a poor prognosis[35], while Medici et.al[36] reported that RAS mutation could predict a limited aggressiveness in PTC. However, only eight RAS-positive samples were involved in Medici et.al's study[36].

The problem is that many researches have been conducted to establish the correlation between certain somatic mutation and clinical characteristics, while much fewer studies were designed in an attempt to investigate the molecular dysregulations caused by

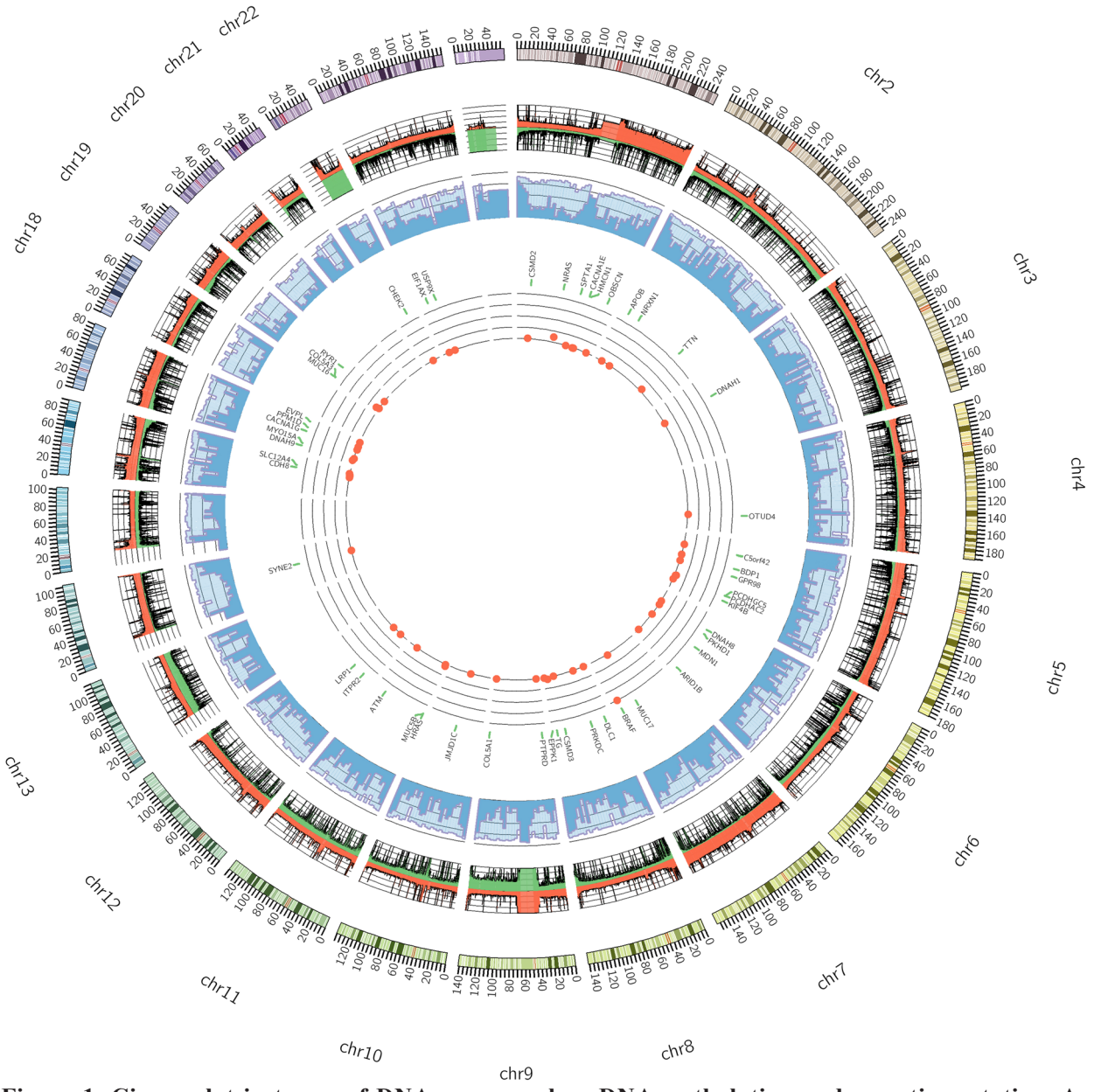


**Figure 1. Circos plot in terms of DNA copy number, DNA methylation and somatic mutation. An ideogram of a normal karyotype is shown in the outermost ring. The next outermost ring represents DNA copy number at corresponding genomic coordinates, calculated by the segment gain or loss (SGOL) scores (red represents amplification and green represents deletion). The next ring represents the amount of DNA methylation. The whole genome was segmented into contiguous 500,000 base pair (bp) bins, and the median (dark blue) and 75th percentile (light blue) of methylation levels of CpGs which could be mapped onto each bin were plotted. The innermost ring is scatter plot illustrating somatic mutation data, genes with mutation rate > 1% were shown in red.**

these distinctive mutations, especially in the era of high-throughput technology. Carcinogenesis is a highly complicated process, therefore besides MAPK and PI3K/AKT canonical pathways proven dysregulated by BRAF and NRAS mutations, there must be other biological and molecular alterations caused by these two mutations, leading to thyroid tumorigenesis. In this study, by virtue of publically available datasets, we were trying to reveal the major genomic and molecular alterations caused by BRAF and NRAS mutations, and identify the potential biological functions exerted by these two mutations, which is essential for understanding what is actually happening underneath their close association with clinico-pathological characteristics of PTC.

## 2. Methods

### *2.1 Data retrieval*

All the genomic and clinical data were downloaded from The Cancer Genome Atlas (TCGA) database, and the ethical approval of TCGA project is granted by National Cancer Institute (NCI). The written informed consent was obtained from all the patients in TCGA database by NCI. The multi-dimensional data of thyroid carcinoma (THCA)-related datasets were all retrieved from the Bioconductor package "RTCGA.rnaseq"

**Table 1. Clinico-pathological analysis of BRAF mutation in 8 clinical factors**

| Characteristic | $BRAF^{MUT}$ | $BRAF^{WT}$ | p |
|---|---|---|---|
| Number(n=397) | 237 | 158 | |
| Mean age ± SD (y) | 47.83±15.46 | 46.39±16.07 | 0.377 |
| Gender | | | 0.310 |
| Female | 174 | 123 | |
| Male | 65 | 35 | |
| Mean tumor diameter ± SD(cm) | 2.91±1.69 | 3.03±1.47 | 0.512 |
| Histological diagnosis | | | $8.126\times10^{-8}$ |
| Classical/usual | 194 | 88 | |
| Follicular + Tall cell | 45 | 70 | |
| Multifocality | | | 1 |
| One lesion | 128 | 82 | |
| More than one lesion | 108 | 70 | |
| Extrathyroidal invasion | | | $3.108\times10^{-6}$ |
| Yes | 91 | 24 | |
| No | 143 | 126 | |
| Lymph node metastasis | | | $8\times10^{-3}$ |
| Yes | 113 | 47 | |
| No | 78 | 63 | |
| Metastases | | | 1 |
| Yes | 5 | 2 | |
| No | 141 | 78 | |

SD: standard deviation.

**Table 2. Clinico-pathological analysis of NRAS mutation in 8 clinical factors**

| Characteristic | $NRAS^{MUT}$ | $NRAS^{WT}$ | p |
|---|---|---|---|
| Number(n=397) | 34 | 363 | |
| Mean age ± SD (y) | 45.32±15.35 | 47.44±15.74 | 0.448 |
| Gender | | | 1 |
| Female | 25 | 272 | |
| Male | 9 | 91 | |
| Mean tumor diameter ± SD(cm) | 2.83±1.64 | 2.97±1.58 | 0.663 |
| Histological diagnosis | | | $1.357\times10^{-4}$ |
| Classical/usual | 14 | 268 | |
| Follicular + Tall cell | 20 | 95 | |
| Multifocality | | | 0.450 |
| One lesion | 21 | 189 | |
| More than one lesion | 13 | 165 | |
| Extrathyroidal invasion | | | 0.050 |
| Yes | 4 | 111 | |
| No | 27 | 242 | |
| Lymph node metastasis | | | $1\times10^{-3}$ |
| Yes | 5 | 155 | |
| No | 20 | 121 | |
| Metastases | | | 1 |
| Yes | 1 | 6 | |
| No | 17 | 202 | |

SD: standard deviation.

clinical information, including 496 samples. Therefore, PTC primary tumor data, including 494 DNA copy number data, 496 methylation data, and 397 somatic mutation data, were used to construct integrative Circos plot via Perl software "Circos plot". For copy number data, Bioconductor package "cghMCR" was used to compute the segment gain or loss (SGOL) scores to quantify common gains/losses by summation of the score in each patient across genomic regions. As for DNA methylation, the whole genome was equally segmented into contiguous 500,000 base pair (bp) bins, and the median and 75th percentile of methylation levels mapped onto each bin were plotted. For somatic mutation data, genes with mutation rate > 1% were shown in scatter plot.

### *2.3 Landscape of major genomic alteration in PTC primary tumors*

Integrated genomic data for 453 PTC samples (columns), including somatic mutations (classified as truncating, in-frame or missense), high-level amplifications (genes with copy number >2) and deletions (genes with copy number<-2). Overall clinical information data for each sample were shown as tracks at the bottom. The percentage of genomic alteration across all PTC samples were labeled at the left of the rows.

### *2.4 Identification of genes with significant alterations between BRAF and NRAS mutations at multi-level*

Differentially expressed mRNAs were identified using two-tailed t test (FDR < 0.01, fold change > 1.5). As for DNA copy number data, Bioconductor package "CNTools" was adopted to process segmentation data into a gene-level matrix according to corresponding genomic location of 28918 genes. Genes with significant copy number variance (CNV) were identified with tow-tailed t statistic test (FDR < 0.01, fold change > 1.5). In methylation analysis, significant hyper-methylated and hypo-methylated genes were also identified with two-tailed t statistic test (FDR < 0.01, $|\Delta| > 0.2$).

### *2.5 Gene Set Variation Analysis (GSVA)*

To summarize the expression of the gene signature representing various immune subpopulations[38], we condensed the expression of the multiple genes into a single gene set enrichment value using GSVA algorithm[39]. Two-tailed t tests was applied to identify the immune subpopulations represented by the significant gene signatures differentially expressed between BRAF and NRAS mutated samples (FDR<0.01,

|fold change|>log1.2).

### *2.7 Statistical analysis*

All statistical analyses were conducted using R project

**Table 3. Clinico-pathological analysis between BRAF-mut and NRAS-mut samples in 3 clinical factors**

| Characteristic | BRAF-mut | NRAS-mut | p |
|---|---|---|---|
| Number(n=271) | 237 | 34 | |
| Histological diagnosis | | | $9.202\times10^{-7}$ |
| Classical/usual | 194 | 14 | |
| Follicular + Tall cell | 45 | 20 | |
| Extrathyroidal invasion | | | $8\times10^{-3}$ |
| Yes | 91 | 4 | |
| No | 143 | 27 | |
| Lymph node metastasis | | | $4.923\times10^{-4}$ |
| Yes | 113 | 5 | |
| No | 78 | 20 | |

†Significant p values were highlighted in bold. SD: standard deviation.

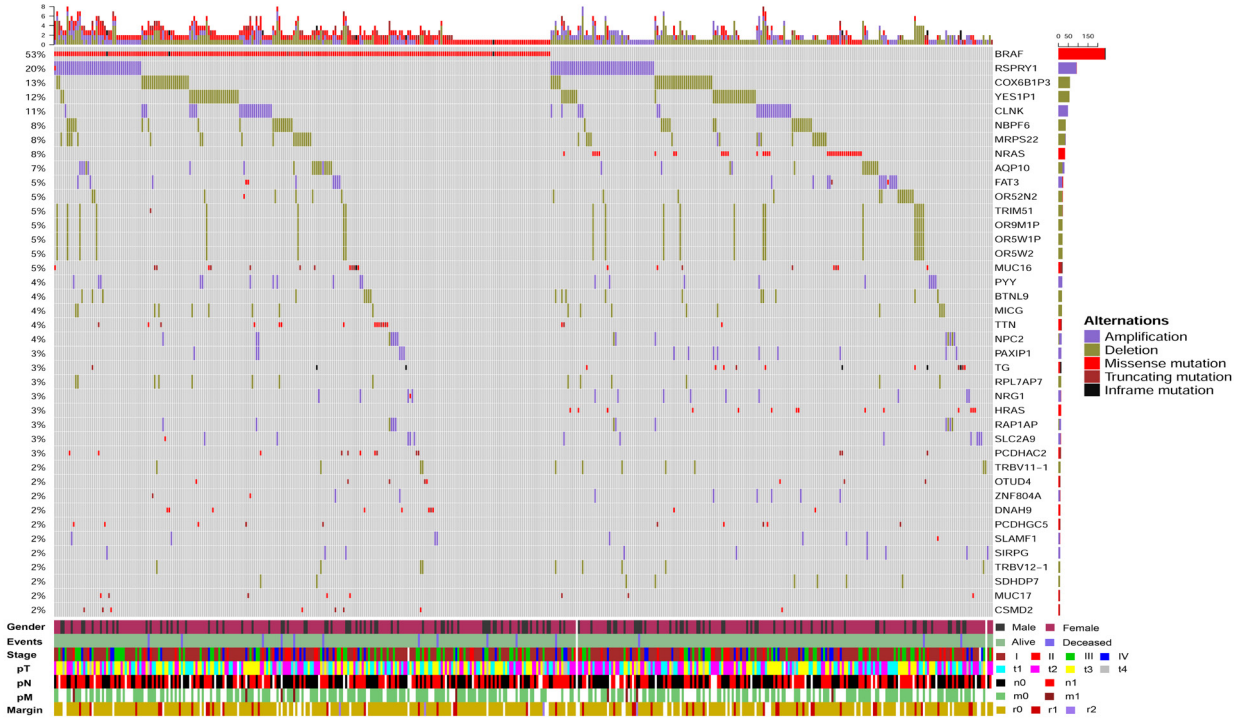


**Figure 2. Landscape of major genomic alteration in PTC primary tumors.**

### *2.6 Pathway activity analysis using RPPA dataset*

Protein expressions measured on a 175-antibody array for 224 PTC samples were used to calculate the pathway activity scores of three groups of primary tumors, including BRAF-mut (BRAFMUTNRASWT), NRAS-mut (BRAFWTNRASMUT) and Neither-mut (BRAFWTNRASWT). We examined the differences in pathway activity scores among these three groups in 9 pathways[40], and two-tailed t test was conducted to identify the pathways significantly differentiated between BRAF-mut and NRAS-mut tumors ($p<0.01$).

software (Version 3.3.1) and Bioconductor (Version 3.0). Gene ontology analysis was conducted using with DAVID Bioinformatics Resources 6.8 (http://david.abcc.ncifcrf.gov/). The Bioconductor annotation package "org.Hs.eg.db" (Version 2.8.0) and "GO.db" (Version 3.6) was used to retrieve T cell related genes. Bar and box charts were all plotted with R package "ggplot2" (Version 2.2.1). Regarding clinico-pathological association analysis, continuous data were expressed as mean±SD. Fisher exact test was used to test the significance of categorical data, and unpaired t tests were adopted for continuous data. A p value $<$ 0.05 was con-

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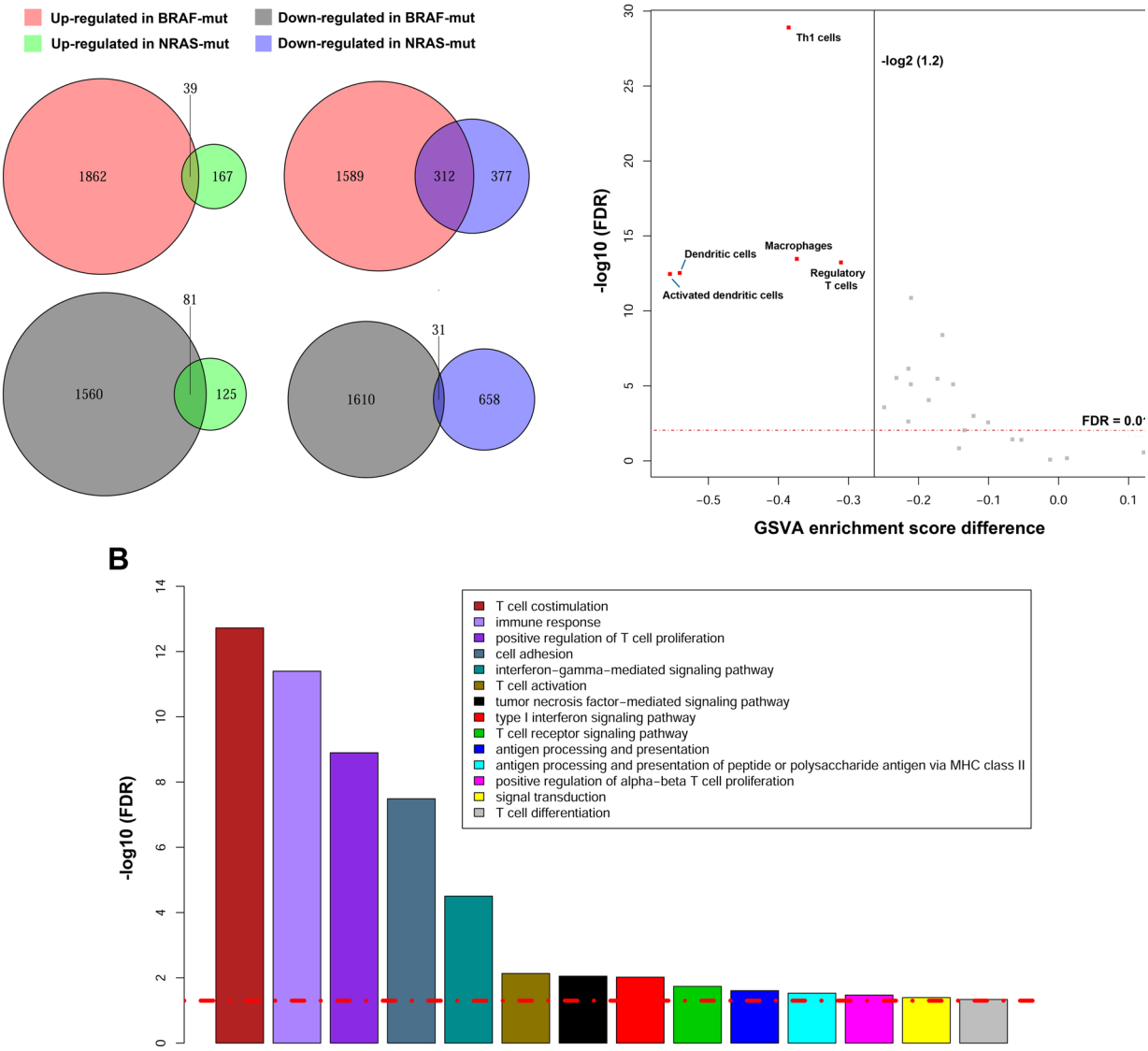


**Figure 3. Reversely expressed mRNAs were closely related to T cell regulation. (A) Abundant differentially expressed mRNAs (including 1901 mRNAs up-regulated and 1641 down-regulated ones in BRAF-mut samples; and 206 mRNAs up-regulated and 689 down-regulated ones in NRAS-mut samples, respectively) were identified in both comparisons with Neither-mut as the control group. mRNAs reversely dysregulated (393 mRNAs) in BRAF-mut and NRAS-mut samples were considerably more than those dysregulated at the same direction (70 mRNAs). (B) GO analysis indicated these 393 reversely expressed mRNAs were significantly associated with T cell regulation, antigen presenting, and immune response. (C) GSVA enrichment score calculated using these 393 mRNAs were significantly different in 5 immune cell subpopulations, including Th1 cells, dendritic cells, activated dendritic cells, macrophages and regulatory T cells.**

sidered as statistically significant.

## 3. Results

### *3.1 BRAF and NRAS were the two most commonly mutated genes in PTC*

As illustrated in Figure 1, profound genomic alterations were formed during the process of carcinogenesis in PTC. To identify the genes afflicted most with genomic alterations, we collected 40 genes with genomic alteration rate ≥2% based on PTC samples with significant high-level CNVs and somatic mutations (Figure. 2). BRAF and NRAS were the two most commonly mutated genes, with the mutation rate 53% and 8%, respectively. Additionally, there were no PTC

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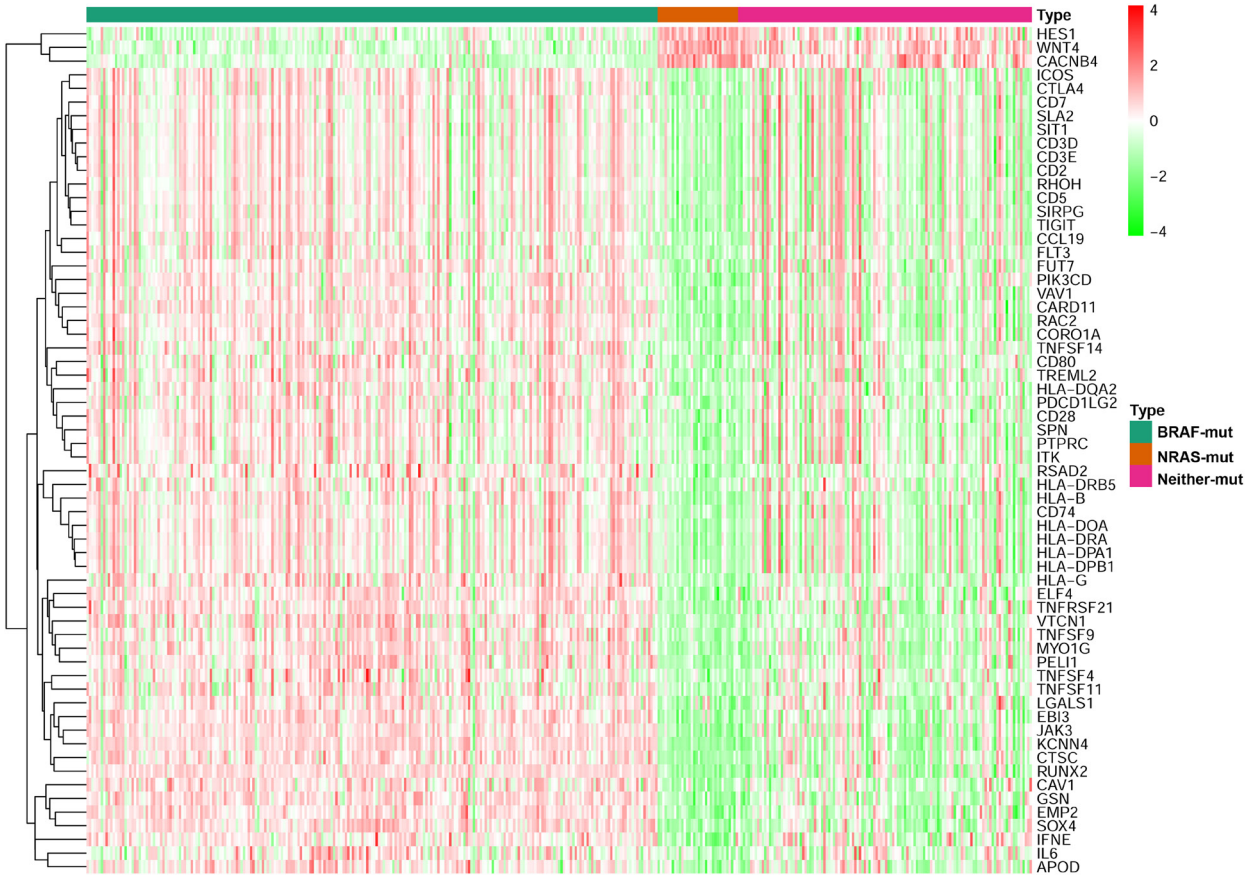


**Figure 4. The 62 T cell related genes reversely expressed in BRAF and NRAS mutated samples. Column represented PTC samples of three subgroups, and rows represented 62 T cell genes. Rows were clustered according to unsupervised clustering algorithm.**

samples with both BRAF and NRAS mutations.

### *3.2 $BRAF^{WT}NRAS^{WT}$ was the intermediate status between $BRAF^{MUT}NRAS^{WT}$ and $BRAF^{WT}NRAS^{MUT}$ in terms of clinico-pathological association*

Table 1 and Table 2 summarized the clinico-pathological association of BRAF and NRAS mutation status, respectively. Eight clinical factors were evaluated, including age, gender, tumor size, histological diagnosis, multifocality, extrathyroidal invasion, lymph node metastasis and distant metastasis. Among these clinico-pathological factors, BRAF and NRAS mutations were both significantly associated with histological diagnosis, extrathyroidal invasion and lymph node metastasis, suggesting there might be overlapping signaling pathways simultaneously affected through BRAF mutation and NRAS mutation (Table 1-2).

Since there were no PTC samples with both BRAF and NRAS mutations at the same time, we divided the primary tumors into three sample groups, i.e. BRAF-mut ($BRAF^{MUT}NRAS^{WT}$), NRAS-mut ($BRAF^{WT}NRAS^{MUT}$) and Neither-mut ($BRAF^{WT}NRAS^{WT}$) samples according to somatic mutation information. Clinico-pathological association analyses were conducted between each pair of these three sample groups in terms of histological diagnosis, extrathyroidal invasion and lymph node metastasis, since these factors were all identified to be significantly associated with both BRAS and NRAS mutation status (Table 3, Table S1-2). The sample distributions of these 3 clinical factors were all significantly different between BRAF-mut and NRAS-mut tumors (all $p < 0.05$, Table 3). In BRAF-mut samples, 194 out of 239 samples (81.17%) belonged to classical histological type, while this percentage was 59.84% (73/122) in Neither-mut samples, and 41.18% (14/34) in NRAS-mut samples. Extrathyroidal invasion was observed in 91 out of 234 BRAF-mut samples (38.89%), while this percentage was 17.09% (20/117) in Neither-mut samples, and 12.90% (4/31) in NRAS-mut samples. Additionally, 113 out of 191 BRAF-mut samples (59.16%) had lymph node metastasis, while the number was 49.40% (41/83) in Neither-mut subgroup, and 20.00% (5/25) in NRAS-mut. Therefore, BRAFWTNRASWT could be regarded as the intermediate status between

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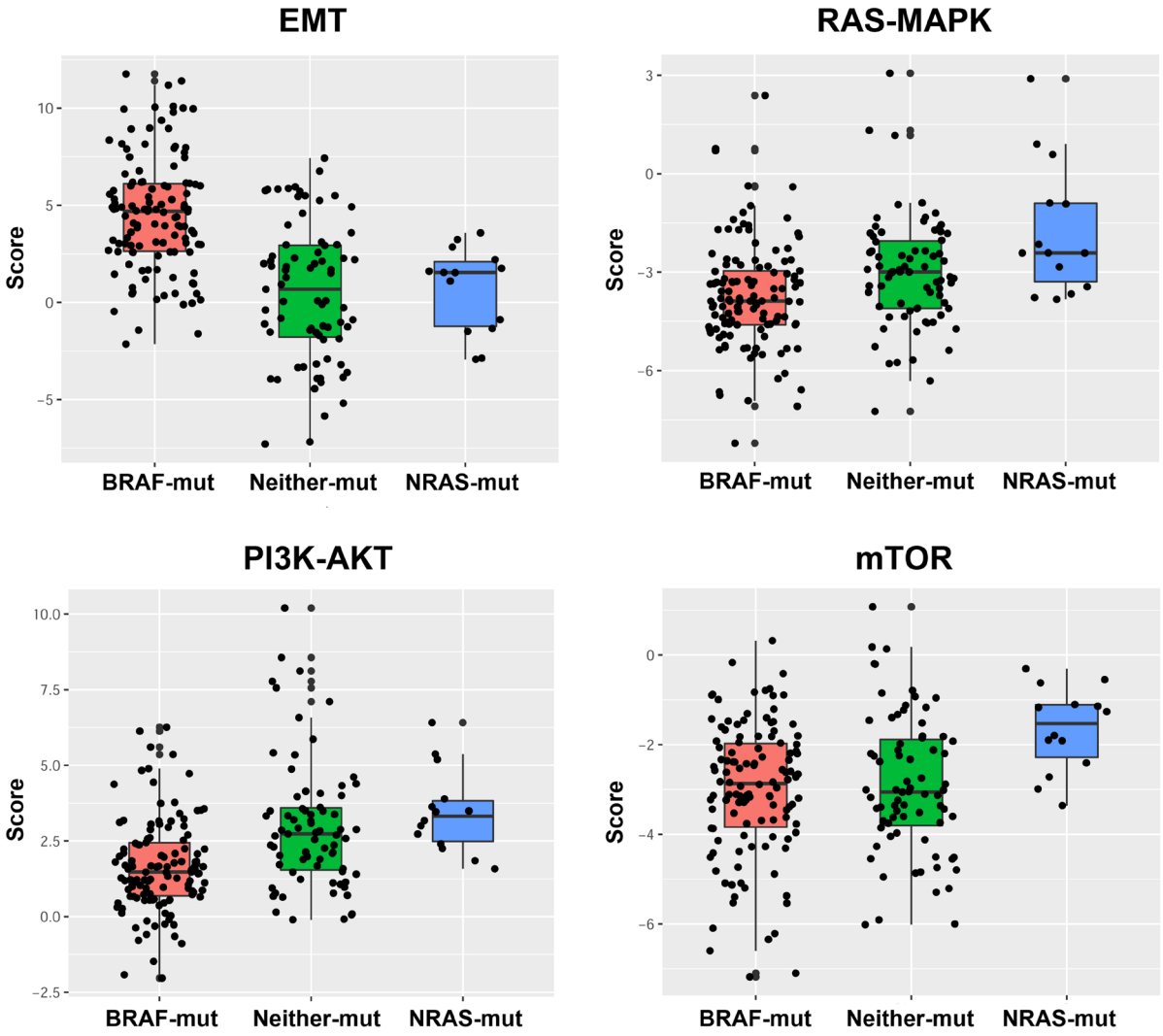


**Figure 5. The activity scores of 4 signaling pathways were significantly different between BRAF-mut and NRAS-mut samples. The activity scores of 4 pathways, including EMT, RAS/MAPK, PI3K/AKT and mTOR pathways were significantly different between BRAF-mut and NRAS-mut samples, and the scores of Neither-mut samples were all generally located between BRAF-mut and NRAS-mut samples.**

BRAFMUTNRASWT and BRAFWTNRASMUT according to the association analyses of these three clinical factors, and the genomic or molecular dysregulations caused by BRAF and NRAS mutation might be oppositely influential in histological type, extrathyroidal invasion and lymph node metastasis, as well as in related signaling pathways of PTC.

### *3.3 Expression differentiation is the major dysregulation caused by BRAF and NRAS mutation*

As indicated in clinico-pathological association analysis, BRAF and NRAS mutations were oppositely associated with PTC clinical characteristics comparing with Neither-mut samples. Therefore, we were trying to identify CNVs, altered methylations and differentially expressed mRNAs, between BRAF-mut and Neither-mut samples, and between NRAS-mut and Neither-mut samples, respectively. Surprisingly, there were neither significantly CNVs (FDR < 0.01, fold change > 1.5) nor altered methylations (FDR < 0.01, $|\Delta| > 0.2$) identified in both BRAF-mut and NRAS-mut samples (both relative to Neither-mut samples). However, abundant differentially expressed mRNAs (including 1901 mRNAs up-regulated and 1641 down-regulated ones in BRAF-mut samples; and 206 mRNAs up-regulated and 689 down-regulated ones in NRAS-mut samples, respectively) were identified in both comparisons (FDR < 0.01, fold change > 1.5, Figure. 3A), suggesting regulating mRNA expression might be the major way for BRAF and NRAS mutation to exert their biological

functions.

Additionally, mRNAs reversely dysregulated (393 mRNAs) in BRAF-mut and NRAS-mut samples (i.e. 312 mRNAs up-regulated in BRAF-mut while down-regulated in NRAS-mut samples, and 81 mRNAs down-regulated in BRAF-mut while up-regulated in NRAS-mut samples) were considerably more than those dysregulated at the same direction (70 mRNAs), suggesting the biological influences exerted by BRAF mutation and NRAS mutation might be opposite with each other, leading to their reverse clinical associations (Figure.3A).

### *3.4 Differentially expressed mRNAs in BRAF-mut and NRAS-mut samples were closely related to T cell regulation*

Furthermore, we conducted Gene Ontology (GO) enrichment analysis with 393 mRNAs reversely dysregulated in BRAF-mut and NRAS-mut samples. Using GO enrichment analysis, conducted with DAVID Bioinformatics Resources, we found that nearly all the enriched GO terms were significantly associated with T cell regulation, antigen presenting, and immune response, suggesting a significant association between these two major mutations and T cell regulation (Figure. 3B). We finally retrieved 62 T cell related genes from BRAF-NRAS reversely expressed mRNAs, while searching through Gene Ontology database (Figure. 4). Additionally, GSVA were used to identify significantly dysregulated gene signatures representing various immune cell subpopulations between BRAF-mut and NRAS-mut samples with mRNA expression profile (Figure. 3C). The result indicated that the GSVA enrichment score were significantly different in 5 immune cell subpopulations, including Th1 cells, dendritic cells, activated dendritic cells, macrophages and regulatory T cells (Figure. 3C, FDR<0.01, |fold change|>log1.2).

Fibronectin was considerably over-expressed in BRAF-mut while under-expressed in NRAS-mut samples

The activity scores of 9 signaling pathways were calculated with the RPPA data in BRAF-mut, NRAS-mut and Neither-mut samples[40], and two-tailed t test was used to identify the pathways significantly dysregulated between BRAF-mut and NRAS-mut samples. There were 4 signaling pathways significantly dysregulated between these two sample groups (p<0.001), including epithelial-to-mesenchymal transition (EMT, p=9.807×10-6), RAS/MAPK (p=0.003), PI3K/AKT (p=2.843×10-4) and mTOR pathways (p=1.110×10-4, Figure. 5). Surprisingly, the activity scores of Neither-mut samples were all generally located somewhere between BRAF-mut and NRAS-mut samples (Figure. 4), suggesting BRAF and NRAS mutations might contribute opposite functions in these biological processes.

Since BRAF and NRAS mutations were reversely associated with extrathyroidal invasion and lymph node metastasis in PTC, we further focused our attention upon EMT process, which involved Fibronectin, Ncadherin, Collagen VI, Claudin7 and Ecadherin 5 proteins (Figure. 6A). We compared the expression level of each protein between BRAF-mut and NRAS-mut samples, and only the level of Fibronectin was significantly different between these two mutations (p=6.492×10-7, Figure. 6A). The level of Fibronectin in Neither-mut was significantly higher than that in NRAS-mut, and lower than that in BRAF-mut samples, indicating its great importance in regulating PTC's invasion and migration under the circumstances of BRAF and NRAS mutation.

Additionally, the mRNA level of fibronectin was significantly and positively correlated with its protein level using 211 PTC samples (p=4.680×10-33, Figure. 6B), and mRNA expression level of Fibronectin showed the similar tendency with that of protein level in three sample groups (Figure. 6C).

## 4. Discussion

Activating mutations of BRAF and NRAS genes were generally perceived to be mutually exclusive in PTC[41,42], and this phenomenon was also confirmed in our study. BRAF and NRAS mutations were the top two mutated genes in PTC samples, also consistent with previous researches[43]. Clinico-pathological association analysis was conducted using 8 clinical factors. The result indicated that both BRAF and NRAS mutation were significantly and only associated with 3 factors, including histological diagnosis, extrathyroidal invasion, and lymph node metastasis. There were only 7 distantly metastatic PTC samples with exact mutational information, so whether distant metastasis is associated with BRAF and NRAS mutations could not be statistically determined in this study. The surprising coincidence of the overlapping 3 factors implied that the downstream genomic or molecular alterations might affect similar signaling pathways, leading to the dysregulations of similar biological processes of PTC cells. Therefore, we further divided PTC samples into three groups, including BRAF-mut, NRAS-mut and Neither-mut subgroups, based on their corresponding mutational status. We believed that Neither-mut samples should be chosen as a control cohort for evaluating the biological and clinical influences generated from these two major mutations, and it is more reasonable to examine the effects caused by one mutation after eliminating the bias conferred by the other. Surprisingly, the pair-wise clinico-pathological association analysis indicated that the distributions of Neither-mut (BRAFWTNRASWT) samples were all

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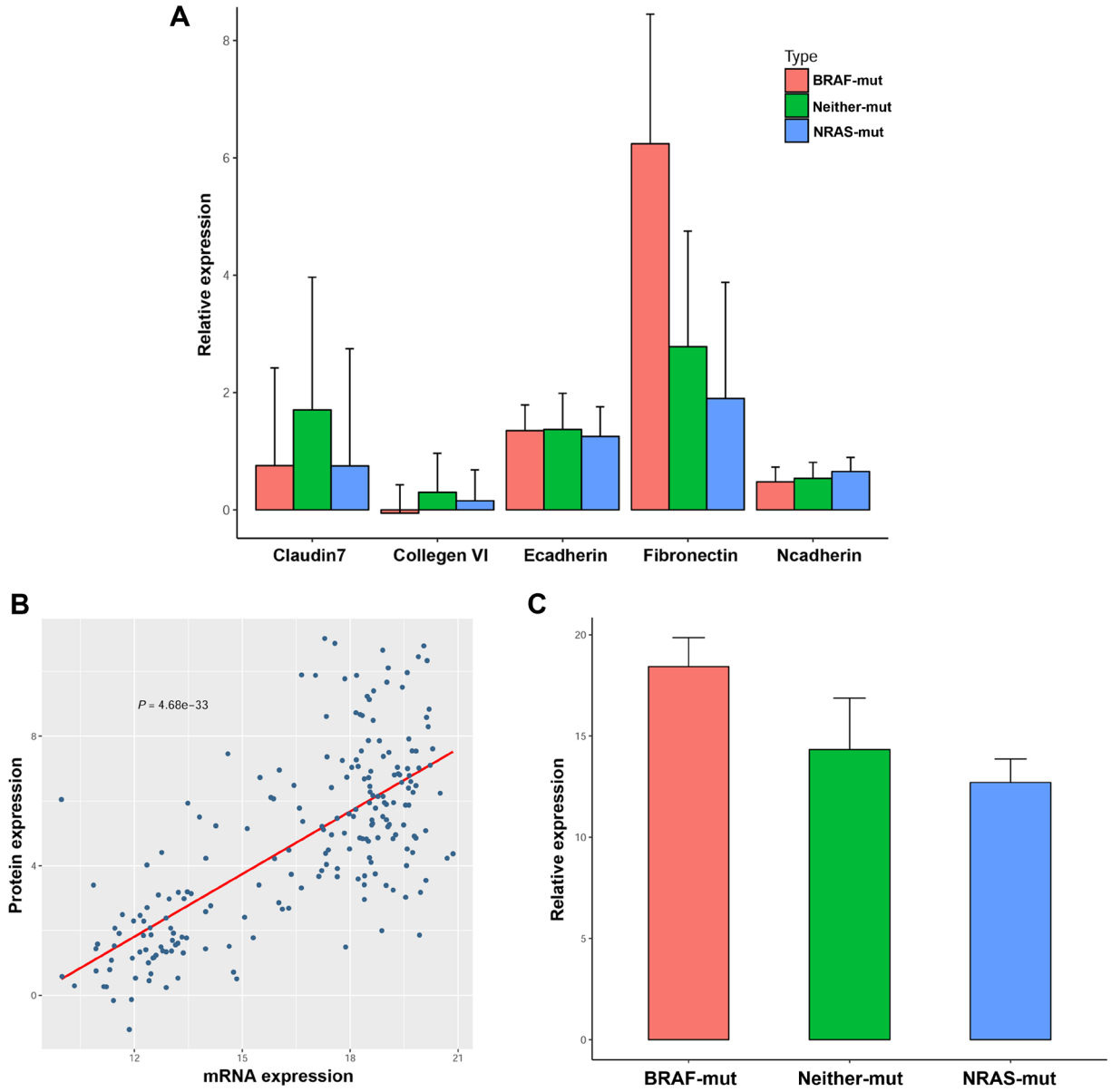


**Figure 6. Both protein and mRNA levels of Fibronectin were strictly following descending order of the BRAF-mut, Neither-mut and NRAS-mut sequence. (A) Bar chart of 5 proteins representing EMT pathway, which involved Fibronectin, Ncadherin, Collagen VI, Claudin7 and Ecadherin. Fibronectin was the only protein responsible for the descending order of EMT pathway score. (B) The mRNA level of fibronectin was significantly and positively correlated with its protein level using 211 PTC samples with both mRNA and protein expression data. (C) mRNA expression level of Fibronectin showed the similar tendency with that of protein level in three sample groups.**

located at the intermediate position between BRAF-mut (BRAFMUTNRASWT) and NRAS-mut (BRAFWTNRASMUT) samples in terms of histological diagnosis, extrathyroidal invasion and lymph node metastasis. This result confirmed that PTCs with NRAS mutation were less aggressive with the comparison to those with BRAF mutation or BRAFWTNRASWT samples. Similar conclusions were also addressed in Medici et.al's study[36]. However, the RAS-positive samples involved in Medici et.al's study was only eight, and this small sample number might probably introduce systematic bias. Additionally, NRAS, KRAS and HRAS mutations were aggregately defined as RAS-positive. Although NRAS, KRAS and HRAS all belong to RAS gene family, the mutations of these three genes might cause different molecular consequences and potentially distinct biological func-

tions since they are different genes located at different genomic positions. Therefore, it is more sensible to take each RAS gene separately into consideration.

Although the association between somatic mutations and clinico-pathological characteristics has been continuously and intensively studies in PTC, the genomic and molecular alterations caused by BRAF and NRAS mutations have received relatively much less attention than it deserves. Understanding the complicated downstream molecular events rendered by these important mutations might be greatly helpful for us to discover potential therapeutic targets in treating PTCs. Over the last two decades, the booming development of high-throughput technology ushered us into a new era, in which the tremendously complicated genomic and molecular mechanism of carcinogenesis could be analyzed in a much more integrative perspective. It is putatively accepted that the process of carcinogenesis is achieved through the extensive dysregulations of many layers, including genomics [majorly somatic mutation and CNV][44,45], DNA methylomics[46,47], transcriptomics[48,49], and so on. Therefore, in this study, multi-layer molecular dysregulations in BRAFMUTNRASWT and BRAFWTNRASMUT (both relative to BRAFWTNRASWT samples) were evaluated, since BRAFWTNRASWT were observed as the intermediate status between BRAF mutation and NRAS mutation in terms of clinical association analysis. No significant CNVs or altered methylations were identified in both comparisons, indicating CNV and methylomic dysregulation was not the molecular consequences of BRAF or NRAS mutation. On the contrary, the abundance of differentially expressed mRNAs implied that transcriptomics, instead of CNV or methylomics, were the major molecular events caused by these two mutations.

We hypothesized that the oppositely dysregulated mRNAs in BRAF-mut and NRAS-mut samples (both comparing with the control cohort, Neither-mut samples) might contain profound biological information of the clinical divergence between these two major PTC mutations. Therefore, we identified differentially expressed mRNAs between BRAF-mut and Neither-mut samples, and between NRAS-mut and Neither-mut samples, respectively. Accordant with our previous hypothesis, mRNAs reversely dysregulated (393 mRNAs) in BRAF-mut and NRAS-mut samples (i.e. mRNAs up-regulated in BRAF-mut while down-regulated in NRAS-mut samples, and mRNAs down-regulated in BRAF-mut while up-regulated in NRAS-mut samples) were considerably more than those dysregulated at the same direction (70 mRNAs). Clinical associations link closely to biological behaviors, which are strictly regulated through highly complex molecular regulatory network that we currently know little about. The number of reversely expressed mRNAs accounts for 84.88% of all the overlapping differentially expressed mRNAs in both comparisons, suggesting the major effects of signaling dysregulations caused by BRAF and NRAS mutations might be opposite to each other. Thus, we focused our attention upon 393 reversely expressed mRNAs in further analysis.

GO analysis indicated that these 393 reversely expressed mRNAs were significantly associated with T cell regulation, antigen presenting and immune response, which was also confirmed by GSVA analysis. As far as we know, there have been no previous study focusing on the relationship between T cell regulation and somatic mutation in PTCs. The undeniable relationship between immune aberration and cancer has been reported and is putatively accepted by cancer research community. Chronic inflammation and immune disorder contribute to nearly 25% of all cancers worldwide[50]. Chronic, or subclinical inflammation has many tumor-promoting influences such as increased proliferation and survival of tumor cells, promotion of angiogenesis and metastasis, disruption of immune response system, and alteration in the response to hormones and chemotherapeutic agents[51]. Immuno-compromised patients, for instance, organ transplant recipients[52,53] or patients with AIDS[54,55], have a remarkably increased risk for tumor formation. Thus, immune-related genes play a very important role in carcinogenesis and need considerable attention in future cancer research upon PTC. Therefore, the distinct clinical associations and outcomes of BRAF or NRAS mutation might be derived from T cell dysregulation. Cancer immunotherapies has achieved a marvelous development in the last decade[56]. For example, extensive T cell infiltration was observed in advanced thyroid cancer, and PD-1 checkpoint blockades were reported to have therapeutic effect in patients with aggressive tumors[57]. However, since the T cell regulatory status between BRAS-mut and NRAS-mut samples are so distinct, that different clinical outcomes would probably be perceived after the same targeted immunotherapy was used on different PTC patients with different mutation statuses. Further studies were urgently needed to compare the different efficacies.

Fibronectin is a multi-modular glycoprotein, which is abundantly existent in extracellular matrix of various connective tissues. A variety of biological functions were reported to be regulated by Fibronectin under physiological circumstances, including cell adhesion, proliferation, wound healing, invasion and migration[58-60]. Fibronectin has been reported to be up-regulated in various types of human malignancies, including anal cancer[61], breast cancer[62], head and neck squamous cell carcinoma[63] and ovarian cancer[64]. During the process of carcinogenesis, Fibronectin is essentially important in regulating tumor progression[65-67], invasion and

metastasis[68-71], and therapeutic resistance[72-74]. Fibronectin was also reported to be closely related to aggressive cellular behaviors in PTC. For instance, its expression is increased in PTC and favors the migration and invasion of cancer cells[75], and closely associated with lymph node metastasis[76,77]. In our study, the mRNA and protein expressions of Fibronectin were both strictly following the descending order in BRAF-mut, Neither-mut, NRAS-mut sequence, and solely responsible for the same descending order of EMT scores, suggesting that Fibronectin might be the critical molecule oppositely dysregulated by BRAF and NRAS mutation and majorly responsible for lymph node metastasis. Recently, effective antibodies and small agents with great affinity to the EDA and EDB domains of Fibronectin have been developed and successfully utilized in preclinical analysis and small clinical trials[78]. Therefore, the targeted drug designed for Fibronectin might be effective in treating PTC patients, especially for those with BRAF mutation.

In summary, we conducted integrative analysis of PTC with the aid of TCGA database. BRAF and NRAS were the two most commonly mutated genes in PTC, consistent with previous investigations. The multi-level genomic dysregulations during carcinogenesis indicated that while looking into the dysregulation of gene expression in cancer, the aberrant patterns of multi-level events should also be paid considerable attention to shed light on the underlying intricate mechanisms of cancer initiation and deterioration. Clinico-pathological association analysis indicated that both BRAF and NRAS mutations were significantly associated with histological diagnosis, extrathyroidal invasion and lymph node metastasis in PTC, and BRAFWTNRASWT samples was observed as the intermediate status between BRAFMUTNRASWT and BRAFWTNRASMUT in terms of their clinical relevance. Neither significant CNVs nor altered methylations were observed in both mutations, while abundant mRNAs were found oppositely dysregulated in BRAF and NRAS mutation samples comparing with BRAFWTNRASWT samples. These oppositely dysregulated mRNAs were significantly related to T lymph cell regulation and immune response. Finally, Fibronectin was found over-expressed in BRAF-mut samples while under-expressed in NRAS-mut samples, probably responsible for the dysregulated EMT process of PTC cells.

## 5. Conclusions

This study found that the mRNA expression patterns were significantly reversed between BRAF and NRAS mutated PTC samples. These oppositely dysregulated mRNAs were significantly related to T lymph cell regulation and immune response. Fibronectin was found over-expressed in BRAF-mut samples while under-expressed in NRAS-mut samples, probably responsible for the dysregulated EMT process of PTC cells.

## Author contribution

(I) Conception and design: Ning An and Xue Yang
(II) Administrative support: Ning An
(III) Provision of study materials or patients: Xue Yang
(IV) Collection and assembly of data: Xue Yang
(V) Data analysis and interpretation: Ning An and Xue Yang
(VI) Manuscript writing: All authors
(VII) Final approval of manuscript: All authors

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Conflict of interest

All authors have completed the ICMJE uniform disclosure form. The authors have no conflicts of interest to declare.

## Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All the genomic and clinical data were downloaded from TCGA database, and the ethical approval of TCGA project is granted by National Cancer Institute (NCI). The written informed consent was obtained from all the patients in TCGA database by NCI.

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