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RESEARCH PAPER

Mutations of genes including *DNMT3A* detected by next-generation sequencing in thyroid cancer

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ABSTRACT

More than 90% of thyroid cancer belongs to the papillary and follicular thyroid carcinomas based on pathological subtypes. Papillary and follicular thyroid carcinoma are generally associated with a good prognosis. In contrast, other pathological subtypes such as poorly-differentiated and anaplastic thyroid carcinoma (PDTC and ATC) have a poor clinical outcome with a short life expectancy. To identify the genetic variations and biomarkers that may potentially distinguish the aggressive form of thyroid cancer, we performed a retrospective analysis of the formalin-fixed paraffin-embedded tumor samples from 50 patients who mainly displayed aggressive thyroid cancer using next-generation sequencing of 416 solid tumor-related genes. We adopted extensive bioinformatic analysis to vigorously remove germline single-nucleotide polymorphism and systematic sequencing errors, and report here that mutation in *DNMT3A* gene was significantly enriched in patients with PDTC or ATC.

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Introduction

Thyroid cancer is a common endocrine malignancy, and it has been divided into several pathological subgroups. Papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) account for approximately 90% of all thyroid cancer cases.[1] PTC and FTC are collectively considered as well-differentiated thyroid cancer (WDTC), and are generally associated with good prognosis with over 10-year survival rate in 90% of patients.[1] Medullary thyroid carcinoma (MTC) is the next dominant subtype, accounting for approximately 3%-5% of all thyroid cancer cases. MTC can be associated with inherited predisposition depending on the mutational status of the rearranged with introduction of (*RET*) proto-oncogene.[1] Although rare, poorly-differentiated thyroid carcinoma (PDTC) and anaplastic (also called undifferentiated) thyroid carcinoma (ATC), show the most aggressive progression with frequently uncontrolled local disease or distant metastasis, and with an extremely short life expectation.[1]

With the recent advancement of DNA sequencing technology, biomarkers have been vigorously evaluated in order to improve disease stratification and prognostic implication in the treatment of thyroid cancer.[2] Aberrant activation of the mitogen activated protein kinase (MAPK) pathway is a frequently mutated signaling pathway in PTC and FTC, with mutations in *BRAF* and *RAS* genes showing the highest prevalence.[3] *BRAF*p.V600E mutation accounts for approximately half of all PTC and FTC patients, and has been demonstrated its prognostic value of recurrence.[4,5] *RET* rearrangement and point mutation are also common in WDTC and MTC, respectively.[3] However, the exact molecular mechanism and genetic characteristics related to PDTC and ATC remain poorly understood.

To better understand the thyroid cancer and identify biomarkers with clinical implication, we retrospectively analyzed the formalin-fixed paraffin-embedded tumor samples of a cohort of 50 thyroid carcinoma patients using next-generation sequencing. As EGFR mutation was detected in FTC with lung metastasis by the amplification refractory mutation system (ARMS) method in our hospital (unpublished), we focused on FTC and those deadly or uncontrolled thyroid carcinoma. We interrogated the exonic regions of 416 genes and the intronic regions of a selected subset of genes that are frequently mutated in solid tumors. Due to lack of matched whole blood sample as a negative control, we deployed a series of bioinformatic algorithms to remove germline single nucleotide polymorphisms (SNP) and sequencing errors. Unfortunately, the EGFR exon 19 in-frame deletion case which was identified by ARMS failed to pass the examination of next-generation sequencing because of the biopsied tumor

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sample was too small. The EGFR mutations were also reported by other groups.[6] We confirmed that *NRAS*p.Q61 and *BRAF*p.V600E is a recurrent mutation in this cohort, but with much less lower prevalence. We also found that *DNMT3A* mutation was significantly enriched in PDTC and ATC, suggesting its potential value in informing personalized treatment for patients with thyroid cancer.

Methods

Patient recruitment

This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University, China. A total of 50 thyroid cancer patients diagnosed and treated in our hospital were recruited to this study from Aug 2012 to Dec 2016. Unstained formalin-fixed paraffin-embedded (FFPE) slides or FFPE scrolls were sectioned from archived FFPE tissue blocks. These cases were reviewed by a pathologist (LCG) to confirm the diagnosis and select areas of tumors for DNA extraction.

DNA extraction and library preparation

All FFPE samples were examined by Nanjing Geneseeq Technology Inc., Nanjing, China for testing following previous described procedures.[7] Briefly, FFPE samples were first de-paraffinized with xylene and DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's protocols. Resulting DNA sample was evaluated using NanoDrop 2000 (Thermo Fisher Scientific) for potential organic solvent contamination, and quantified using Qubit 2.0 dsDNA HS Assay (Life Technologies) following manufacturer's instructions.

Sequencing libraries were prepared using KAPA Hyper Prep Kit (KAPA Biosystems) following manufacturer's recommended protocols. Briefly, 1 μg of DNA, if necessary, mechanically sheared into 350bp fragments using Covaris M220 instrument, was processed sequentially by end-repairing, A-tailing, adaptor ligation, and PCR amplification. Purification and size selection was performed using AMPure XP beads.

Hybridization capture and sequencing

Different libraries with unique sample indices were pooled together in desirable ratios for up to 2 μg of total library input. Human cot-1 DNA (Life Technologies) and xGen Universal blocking oligos (Integrated DNA Technologies) were added as blocking reagents. Customized xGen lock down probes (Integrated DNA Technologies) targeting the exonic regions of 416 cancer-relevant genes and selected intronic regions.[7] The capture reaction was performed with the Nimble GenSeqCap EZ Hybridization and Wash Kit (Roche) and Dynabeads M-270 (Life Technologies). Captured libraries were on-beads amplified with Illumina p5 (5′ AAT GAT ACG GCG ACC ACC GA 3′) and p7 primers (5′ CAA GCA GAA GAC GGC ATA CGA GAT 3′) in KAPA HiFiHotStartReadyMix (KAPA Biosystems). The post-capture amplified library was purified by AMPure XP beads and quantified by qPCR using the NEB Next Library Quantification kit (New England Biolabs). The target-enriched library was then sequenced on HiSeq 4000 NGS platforms (Illumina) according to the manufacturer's instructions with an anticipated average depth of coverage of approximately 700X.

Sequence data processing

After performing demultiplexing by bcl2fastq, Trimmomatic was used for FASTQ file quality control.[8] Leading/trailing low quality (quality reading below 15) or N bases were removed. Reads from each sample were mapped to the human reference genome hg19 using Burrows-Wheeler Aligner (BWA-mem, v0.7.12).[9,10] Local realignment around indels and base quality score recalibration were applied with the Genome Analysis Toolkit (GATK 3.4.0).[11] PCR duplicates were removed by Picard (available at: https://broadinstitute.github.io/picard/). VarScan2 was employed for detection of single nucleotide variants[12] and insertion/deletion mutations with the following settings: minimum read depth = 20, minimum base quality = 15, minimum variant allele frequency = 0.01, minimum variant supporting reads = 5, variant supporting reads mapped to both strands, and strand bias no greater than 15%. CNVkit was used for identification of copy number variants,[13] with a segment log2 ratio cutoff of 0.32 for copy number gain and −0.41 for copy number loss.

Variant filtering and annotation

The vcf files generated by VarScan were annotated using ANNOVAR[14] against the following database: dbSNP (v138),[15] 1000Genome[16,] ESP6500 (available at: http://evs.gs.washington.edu/evs/), ExAC,[17] CG46, COSMIC (v70),[18] ClinVAR,[19] and PolyPhen2.[20] Mutations that did not result in non-synonymous or frameshift, or mutations that was not documented in ClinVAR or COSMIC were remove. Mutations showing 1% or higher frequency in the 1000 Genome, ESP6500, ExAC, or CG46 were removed as well. The result mutation lists were filtered through an internally maintained list of recurrent sequencing errors. Mutations occurred within the repeat masked regions while not annotated by COSMIC or ClinVAR were also removed. Remaining mutations were reverse-referenced against the other samples (non-thyroid cancer samples and whole blood samples) sequenced within the same flow-cell. We removed variant that is detected within more than 20% of the non-thyroid cancer samples or within more than 10% of the whole blood samples.

Statistical analysis

Statistical analysis was performed using the R Programming Language.[21] When comparing our PTC and FTC patient data with TCGA results, Fisher's exact test was performed. When comparing patient demographics, Freeman-Halton extension of Fisher's exact test and Cox proportional hazards model was performed. For genes whose mutation was detected in more than 3 samples (43 genes), Freeman-Halton extension of Fisher's exact test was performed to identify significantly mutated genes within subgroups of the patients. Multiple test correction was performed using false discovery rate.

Results

Patient demographics

Fifty thyroid cancer patients diagnosed and treated in our hospital between Aug 2012 and Dec 2016 were included in this study. Each patient was diagnosed with either PTC with distant metastasis during initial assessment, or had FTC, MTC, PDTC, ATC at initial diagnosis. Because of the selection process, half of the cohort was MTC, PDTC and ATC, deviating from the reported statistics. Consistent with the literature, females have a higher incidence of thyroid cancer than males (Table 1). However, no significant difference was detected in the disease pathological type distribution (Fisher's exact test, p = 0.610) and disease stage (WHO 7th edition) distribution (Fisher's exact test, p = 0.073) between genders. Most of (45/50) patients received partial or totalthyroidectomy as the standard treatment protocol. Seven patients with metastatic tumor to the lung (2), brain (2), thoracic vertebra (2) and skull (1) were diagnosed based on the biopsies. Following surgery or biopsy, four patients received radioactive iodine therapy and two patients received chemotherapy while two patients received both radioactive iodine and chemotherapy treatment. Samples including 43 from thyroid tissue and 7 from distant metastases were meeted the criteriaused for analysis by next-generation sequencing.

Mutation profile

We performed targeted sequencing of the exonic regions of 416 solid tumor related genes and the intronic regions of a subset of the genes using Illumina sequencing platform on the archived FFPE samples of our cohort. Regrettably, due to those patients who failed to thrive, we could not collect the matched whole blood samples as a negative control to filter patient specific germline mutations. To circumvent this shortcoming, we screened the detected single nucleotide variants (SNV) and small insertion/deletion (Indel) through the following criteria: 1) the mutations may not demonstrate more than 1% population frequency within the 1000 Genome, ESP6500, ExAC, and CG46 databases; 2) the mutations may not be detected within more than 20% of the non-thyroid cancer samples or within more than 10% of the whole blood samples sequenced within the same flow cell; 3) remove any mutation if all detected incidents were below 2% variant allelic frequency; 4) rescue any mutations removed in the previous steps if it is documented within the COSMIC or ClinVAR databases. For copy number variants (CNV), we set the cutoff of Log2 ratio for calling CNV gain and loss to be 0.32 (Log2 of 1.25) and −0.41 (Log2 of 0.75), respectively. We also removed CNV fragments longer than 5,000,000 base pairs to focus our analysis on focal copy number aberrations.

We obtained a total of 305 SNV, 78 Indel, and 167 CNV after applying the aforementioned filters. Figure 1 displayed the mutational profile of the analyzed cohort. Due to limited sample sizes, we grouped PTC and FTC as WDTC, and PDTC and ATC as LDTC for downstream analysis. *NRAS* (13), *RET* (10), *TP53* (9), *BRAF* (6), and *HRAS* (6) genes remain the most frequently mutated genes, with number in brackets indicating the number of patients carrying the mutations. Three of the four incidences of *BRAF* SNV were c.1799T>A, p.(V600E) mutations, while the other one was a c.1790T>A, p.(L597Q) mutation. Among the 21 mutational events occurred on *NRAS, HRAS*, and *KRAS*, 19 involves the glutamine at codon 61 and 2 involves the glycine at codon 13, consistent with previously reported bias. [22]

We cross-referenced our mutational profiles against the Cancer Genome Atlas (TCGA) database. Interestingly, among the 504 available thyroid cancer (THCA) patients documented in TCGA, 235 of 241 *BRAF* mutations occurred at p.V600E, among which only 12 were detected in FTC patients. None of the mutations within *NRAS, TP53*, and *BRAF* genes were detected in MTC patients, further supporting that MTC display a distinctive mutational profile from the other subtypes. By applying a 2 × 3 extension of Fisher's exact test to the genes whose mutations were recurrently detected in at least 3 patients, we identified that *AMER1* mutation was significantly enriched in MTC and LDTC patients, while *DNMT3A* mutations were significantly enriched in LDTC patients.

Table 1. Summary of patient demographics, thyroid carcinoma subtypes and clinical staging.

		Cases	Significance (Cox Proportional Hazards model)
Gender	Female	31	p = 0.039 *
	Male	19	
Age	<60	24	p = 0.139
	≥ 60	26	
Stage	I	11	
	II	9	p = 1.000
	III	9	p = 0.998
	IVA	9	p = 0.998
	IVB	2	p = 0.998
	IVC	10	p = 0.998
Pathology	Papillary TC	2	
	Follicular TC	23	p = 0.130
	Medullary TC	14	p = 0.193
	Poorly Differentiated TC	4	p = 0.214
	Anaplastic TC	7	p = 0.749

* *p*-value <0.05. TC: Thyroid Carcinoma

Survival analysis

Survival analysis of the patient overall survival (OS) was then applied to our dataset. Consistent with established statistic data, three groups of patients displayed significant difference in survival pattern (p = 0.002), with PDTC and ATC being the group showing worst outcome (Figure 2A). We then extended the analysis for each gene by comparing the OS of the patients with mutations within the gene to that of the patients with wild type. We found that *NRAS* mutations were significantly associated with a poor OS, but the significance was lost after multiple test correction (Figure 2B). We also found that mutations in *TP53, BRAF, DNMT3A*, and *GNAS* were significantly associated with the poor survival (p < 0.05, FDR<0.1) (Figure 2C-F).

Discussions

Thyroid cancer is the most prevalent type of endocrine neoplasm. Molecular pathology has greatly been developed in recent years, and it has been applied in the diagnosis and guideline for the

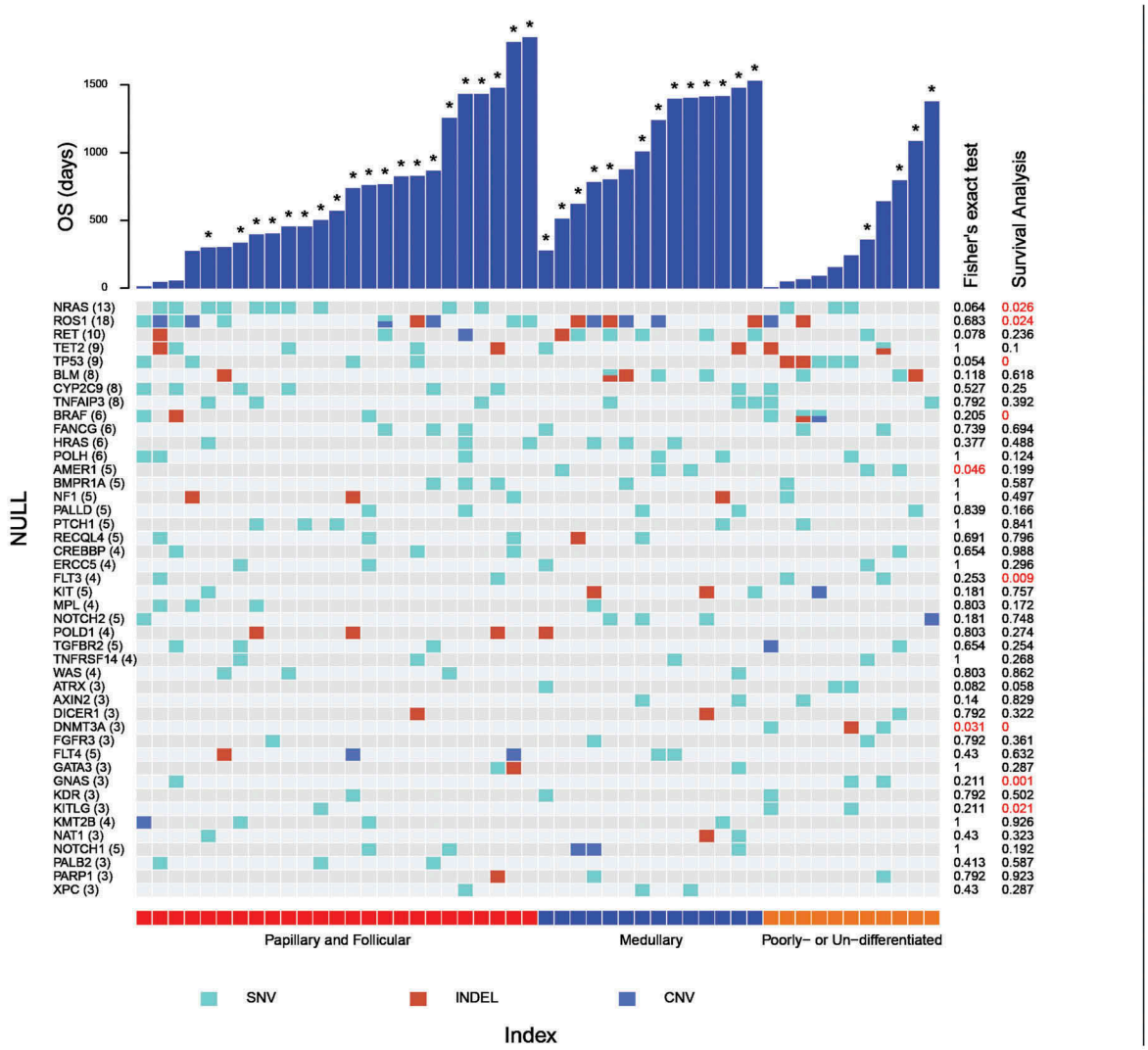

Figure 1. Mutational profile of aggressive thyroid cancer patients. Each column represents an individual patient. Only genes whose mutations were detected in at least 3 patients were shown. The numbers in brackets following each gene name indicates the number of patients carrying the mutation. Overall survival of patients was plotted as bar graph, with asterisk marking the patients who still thrive at last revisit. The p-value of Freeman-Halton extension of Fisher's exact test and survival analysis were listed on the right side with red color highlighting p-values < 0.05.

treatment.[23] The diagnosis of thyroid cancer has been heavily relied on genetic analysis as an extra adjunct of morphology and immuno-histochemical staining results. For instance, genetic alterations defining subtypes in thyroid cancer include *RET* and *NTRK* mutation in subsets of PTC[24,25] and *PAX8-PPARγ* in FTC.[26] In general, molecular transcripts have been respectively associated with thyroid cancer, such as *BRAF p.V600E, RAS, DNMT3* mutation, *RET, NTRK1, NTRK3, ALK, PAX8* gene rearrangement, and other related oncogenes.[27–30] Besides diagnostic and genetic values, the detection of genetic changes in thyroid cancer has been becoming an effective way to search for new therapeutic targets. Recently, the *RET* inhibitors, including vandetanib[31] and cabozantinib,[32] have been approved by the US Food and Drug Administration (FDA) to treat metastatic MTC because they have anti-proliferative role in *RET*-mutant MTC. Also, the sensitivity of vandetanib has been observed in PTC with *RET* mutation.[33] But little is known about oncogene driven mutation in aggressive thyroid cancer. Therefore, efforts to target several other molecular alterations are still under way.

EGFR 19del was first detected in a FTC female patient with ARMS method in our hospital in 2015. EGFR mutation often occurred in lung adenocarcinoma, especially in Asian female without smoking.[34] Target therapy with small molecular EGFR tyrosine kinase inhibitor has achieved amazing outcomes in these EGFR mutation non-small cell lung cancer patients, but little is reported in thyroid carcinoma.[6,35] It is interesting that the PFS is nearly 11 months in this patient treated with EGFR tyrosine kinase inhibitor. As far as we know, this is the first reported clinical efficacy of EGFR TKI in FTC with EGFR mutation. Even only one case with EGFR mutation was found in our study, it is also suggested that EGFR mutation should be detected especially in uncontrolled thyroid cancer in clinical practice as only 50 thyroid cancer patients were recruited in our study. In this study, we retrospectively analyzed the archived FFPE tumor samples from 50 thyroid cancer patients with next-generation sequencing. Despite lacking the matched whole blood sample, which may constitute aminor setback to the analysis, we performed

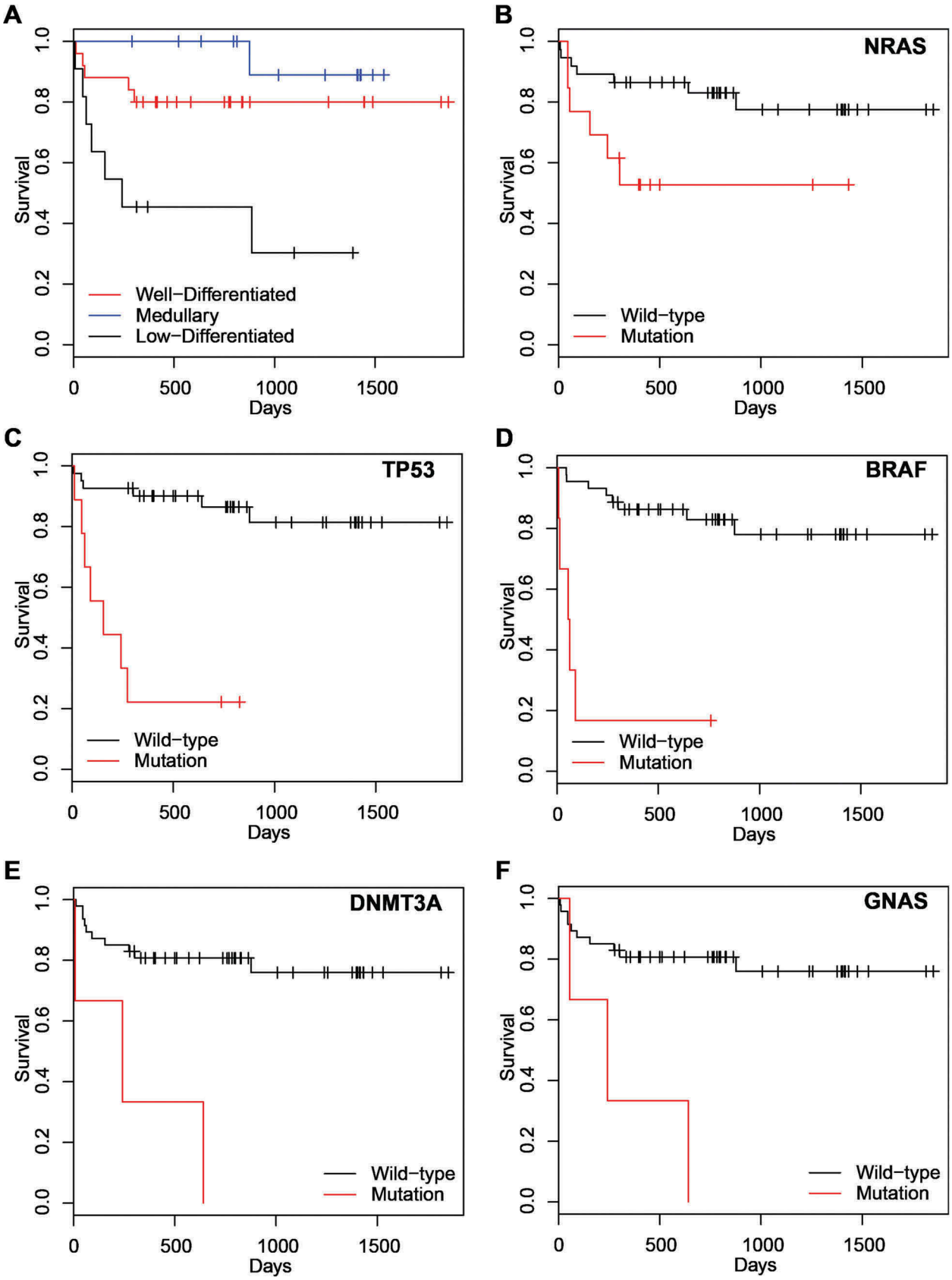

Figure 2. Survival Plot. Patient grouping is based on **A)** pathology subtypes and mutational status, **B)** the *NRAS* gene, **C)** the *TP53* gene, **D)** the *BRAF* gene, **E)** the *DNMT3A* gene, and **F)** the *GNAS* gene.

extensive bioinformatic algorithms and vigorously removed contamination from patient specific germline mutations. The filtering process is also capable of suppressing systematic sequencing error unique to the flow cell, as it evaluates each mutation against the samples of other cancer types and the unrelated whole blood control samples within the same

sequencing run. Nevertheless, it should be pointed out that bioinformatic filtering is not capable of replacing matched normal sample control at removing germline mutations. Indeed, 201 of the 305 SNV mutations had a variant allelic frequency between 40% and 60% or greater than 98%, highly reminiscent of that of a germline mutation. The *AMER1* mutations enriched in MTC and LDTC were an example, all 5 incidences of *AMER1* mutation were c.85G>A and were documented in dbSNP as rs138399473. The three *GNAS* mutations identified through survival analysis also falls into this category.

We noticed that although *BRAF*p.V600E was a frequently mutated gene in WDTC and LDTC, its recurrence frequency was much lower than the reported frequency in TCGA (Fisher's exact test, $p < 0.001$). This is likely a consequence of the fact that FTC and MTC are the principal subtypes of our cohort. Indeed, a closer examination of TCGA data showed that *BRAF*p.V600E has a much lower prevalence in FTC (12 incidences in 102 cases) than PTC (192 incidences in 358 cases) (Fisher's exact test, $p < 0.001$), while several published work collectively supported that *BRAF* p.V600E is rare in MTC.[36–38] Among the three *RAS* gene family members, most of the mutations occurred at codon Q61 instead of the classical codon G12 or G13. This observation supports the previous report, and suggests a mutational profile unique to the cancer type.[22]

By dividing our cohort into WDTC, MTC, and LDTC and applying Freeman-Halton extension of Fisher's exact test, we also identified *DNMT3A* mutation to be significantly enriched in our LDTC patients. Regrettably, the statistical significance is lost (FDR = 0.6) after multiple test correction, and survival analysis suggests that mutation in *DNMT3A* is significantly associated with short life expectancy in LDTC patients. This gene encodes a DNA methyltranserase that is essential to maintain the methylation status of the genome. Aberrant methylation of promoter region of the DNA mismatch repair gene hMLH1 has been associated with the development of *BRAF*p.V600E mutation in PTC.[39] To our knowledge, *DNMT3A* mutation was only previously reported as a recurrently mutated gene in a Saudi Arabia cohort of 886 PTC patients (1.5% recurrence) and was associated with adverse parameters such as extra-thyroidal extension and late-stage in PTC. Given the limited PTC patients in our cohort, it is not surprising that *DNMT3A* mutations were only detected in our LDTC patients. Our findings suggest that *DNMT3A* can be explored as a potential predictive biomarker or therapeutic target for prognosis and treatment of thyroid cancer.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with ethics committee of the First Affiliated Hospital of Soochow University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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