Genomic alterations in fatal forms of non-anaplastic thyroid cancer: Identification of *MED12* and *RBM10* as novel thyroid cancer genes associated with tumor virulence

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Abstract

Purpose—Patients with anaplastic thyroid cancer have a very high death rate. In contrast, deaths from non-anaplastic thyroid cancer are much less common. The genetic alterations in fatal non-anaplastic thyroid cancers have not been reported.

Experimental Design—We performed next-generation sequencing of 410 cancer genes from 57 fatal non-anaplastic thyroid primary cancers. Results were compared to The Cancer Genome Atlas study (TCGA study) of papillary thyroid cancers (PTC) and to the genomic changes reported in anaplastic thyroid cancer (ATC).

Results—There was a very high prevalence of *TERT* promoter mutations, comparable to that of anaplastic thyroid cancer, and these co-occurred with *BRAF* and *RAS* mutations. A high incidence of chromosome 1q gain was seen highlighting its importance in tumor aggressiveness. Two novel fusion genes *DLG5-RET and OSBPL1A-BRAF* were identified. There was a high frequency of mutations in *MED12* and these were mutually exclusive to *TERT* promoter mutations and also to *BRAF* and *RAS* mutations. In addition, a high frequency of mutations in *RBM10* were identified and these co-occurred with *RAS* mutations and *PIK3CA* mutations. Compared to the PTCs in

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TCGA, there were higher frequencies of mutations in *TP53, POLE, PI3K/AKT/mTOR* pathway effectors, *SWI/SNF* subunits, and histone methyltransferases.

Conclusions—These data support a model whereby fatal non-anaplastic thyroid cancers arise from well-differentiated tumors through the accumulation of key additional genetic abnormalities. The high rate of *TERT* promoter mutations, *MED12* mutations, *RBM10* mutations and chromosome 1q gain highlight their likely association with tumor virulence.

Keywords

Non-anaplastic; Thyroid cancer; Next-generation sequencing; MED12; RBM10

Introduction

Patients diagnosed with anaplastic thyroid cancer (ATC) have a very high death rate. Such patients have a mean survival after diagnosis of only 6 months. We have recently reported the genomic hallmarks of ATC showing a very high incidence of *TERT* promoter mutations in 73% of cases with a co-occurrence with either *BRAF* or *RAS* mutations (1). We also identified a high rate of mutations in *TP53* (73%) as well as a higher frequency of alterations in genes such as *EIF1AX* (9%), *PIK3CA* (18%), and *ATM* (9%) as compared to those reported by The Cancer Genome Atlas Network (TCGA Network) study of well-differentiated papillary thyroid cancer, which showed a low frequency of somatic alterations (2).

Non-anaplastic thyroid cancers (NAT) derived from thyroid follicular cells comprise well differentiated (WDTC) and poorly differentiated thyroid cancer (PDTC). Deaths from WDTC are extremely rare occurring in 1–2%, however because these tumors are the most common form of the disease, this small percentage represents a significant fraction of patient dying of thyroid cancer. Deaths from PDTC are more common, occurring in 30% of patients (3). The genomic characteristics of fatal cases of non-anaplastic thyroid cancers (FNAT) has not been reported before. We hypothesized that such cancers may harbor genetic similarities to ATC. The objective of our study was to report the genetic alterations in fatal cases of non-anaplastic thyroid cancer and compare their molecular profile to that of ATC and to the TCGA landscape of PTCs. Fatal cases of non-anaplastic thyroid cancer are invariably refractory to radioiodine therapy, and traditional chemotherapy and radiotherapy are of marginal benefit (4). Two multikinase inhibitors, sorafenib and lenvatinib, have been approved for treatment of radioiodine refractory non-anaplastic thyroid cancer. Other drugs are currently being tested in early human clinical trials (5) but these efforts are hindered by the lack of knowledge on the genomics of fatal cases of non-anaplastic thyroid cancer. The identification of the genetic alterations in these cancers will therefore have major implications both in our ability to identify those rare patients at high risk of death and also to develop novel drugs which target the pathways responsible for their poor prognosis.

Methods

Patients and tumor samples

After IRB approval, patients with fatal non-anaplastic thyroid cancer (FNAT) were identified from a database of 3774 patients who had primary surgery treatment at Memorial Sloan Kettering Cancer Center from 1985 to 2010. 86 (2.3%) patients were identified who had either died of disease or died with disease. Of these, paraffin embedded tissue blocks from primary tumors were available on 57 (66%) patients. Following Institutional Review Board (IRB) approval, tumor and matched normal (non-neoplastic normal tissue) specimens were obtained and then hematoxylin and eosin stained tumor sections were independently re-evaluated by head and neck pathologists (R.A.G; D.L.C; BX). Tumors were then classified into poorly differentiated thyroid cancer (PDTC), as defined by histological and/or immunohistochemical evidence of follicular cell differentiation and presence of tumor necrosis and/or ≥5 mitoses per 10 high-power fields (×400) (6) and into well differentiated thyroid cancer (WDTC). Patients with well differentiated thyroid cancer were further classified into follicular carcinoma, Hurthle cell carcinoma and the different histological subtypes of papillary thyroid carcinoma such as classical, follicular variant and tall cell variant. Patient demographics, tumor histology, treatment and outcomes were determined by retrospective review of patient charts. Tumors were staged according to the 7th edition of the AJCC staging manual.

Sequencing platform and variant calling

The dataset comprised 35 PDTC and 22 WDTC tumor samples. All 57 tumors were sequenced using the MSK-IMPACT platform, a deep-coverage, targeted next-generation sequencing (NGS) assay encompassing 410 cancer-related genes and approved for clinical use by the NY State Department of Health (7). Of the 35 PDTC, 20 had previously been sequenced using an earlier iteration of MSK-IMPACT comprising 341 genes and reported as part of a cohort of 84 PDTC (1). The MSK-IMPACT (Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets) assay is a next-generation sequencing (NGS) assay approved for clinical use through CLIA (Clinical Laboratory Improvement Amendments) by the Centers for Medicare and Medicaid Services (8). MSK-IMPACT is optimized for DNA extracted from low-input formalin-fixed, paraffin embedded (FFPE) samples. The assay is designed to detect single nucleotide variants (SNVs), indels, copy number variants (CNVs) and structural variants in genes that are functionally relevant to cancer and/or clinically actionable targets. The current assay uses hybrid capture technology (NimbleGen SeqCap EZ library custom oligo) to perform deep (>200×) sequencing (Illumina HiSeq 2500) of all 5781 exons and selected introns of 410 cancer genes, including canonical and selected non-canonical transcripts, the *TERT* promoter region, and 33 introns of 14 rearranged genes (Supplementary Table 1). The panel includes 1042 tiling probes covering single nucleotide polymorphisms (SNPs), allowing genotyping to ensure tumor-normal matching, identify contaminating DNA, and serve as a low-density SNP array for CNV analysis. MSK-IMPACT has been extensively validated.

Copy number aberrations were identified by comparing sequence coverage of targeted regions in a tumor sample relative to a standard diploid normal sample (7). To call allele-

specific somatic DNA copy number we also applied an integrated pipeline called FACETS (9) to Tumor/Normal pairs of bam files according to authors' recommendations. The bam files were processed to generate a read count matrix for all the potentially polymorphic sites from dbSNP/1000-genome database as well as pseudo SNPs to account for regions that have large gaps between consecutive SNPs. The read counts are then used to compute the GC-corrected normalized log-ratio of tumor to normal read depths for total copy number and log odds ratio from cross tabulating the tumor and normal reads into ref and alt alleles for loci that are heterozygous in the germline. These are then segmented jointly to obtain the regions of constant allele specific copy numbers and the segmented data used for allele specific integer copy number calls as well as cellular fractions.

Results

Patient, tumor, treatment and outcome characteristics (Table 1)

Of 57 patients, 52 (91%) patients were over 45 years of age and 32 (56%) were female. The majority of patients had advanced stage disease; 53 (92%) had pT3 or T4 tumors, 34 (60%) gross extra thyroidal extension and 31 (54%) had central or lateral neck metastases. 30 (53%) patients presented with distant metastatic disease. 35 patients had poorly differentiated thyroid cancer and 22 had well differentiated thyroid cancer of whom 2 were classical papillary thyroid cancer (PTC), 2 follicular variant of PTC, 1 micro PTC, 1 PTC with tall cell features, 12 tall cell variant of PTC and 4 Hurthle cell carcinoma. 52 patients were treated by total thyroidectomy and 5 had less than total thyroidectomy (4 lobectomy and 1 subtotal thyroidectomy). The cause of death was distant metastatic disease in 51 patients, loco regional and distant disease in 3 patients, and loco regional disease in 3 patients. The median time to death was 52 months.

Somatic mutations

Frequently altered genes (Figure 1)—There was a high prevalence of telomerase reverse transcriptase (*TERT*) promoter mutations occurring in 60% of patients (PDTC 60%, WDTC 60%). This high prevalence is comparable to 73% of ATC (1) and far higher than the 9% of PTCs from TCGA (2). *TERT* mutations co-occurred with *BRAF* mutations (18/26 p<0.001). They also showed a trend to co-occurrence with *RAS* mutations (10/16) and *EIF1AX* mutations (5/7). *TERT* mutations were mutually exclusive with *TP53* mutations (p=0.08) and with *MED12* mutations (p=0.04), consistent with alternate pathways toward progression to FNAT.

BRAFV600E mutations were present in 40% and mutations in *NRAS* and *HRAS* occurred in 25% and 4% respectively. *RAS* mutations were mutually exclusive with *BRAF* and gene fusions (Figure 1). Mutations in the eukaryotic translation initiation factor *EIF1AX*, reported in 1% of PTCs (2), occurred in 12% of FNAT (Figure 1 and Figure S1A), and were strongly associated with *RAS* (p<0.001, Figure S1B). *EIF1AX* mutations clustered in two regions: the N-terminal domain, as also observed in uveal melanomas (10), or at a unique splice acceptor site between exons 5 and 6 (p.A113splice). This splice site is unique to thyroid cancer and also occurs with high frequency in anaplastic thyroid cancer. It results in a 12–amino acid in-frame deletion (1).

Novel genes altered in fatal nonanaplastic thyroid cancer—Mutations of the gene *MED12* were seen in 14% of patients and all *MED12* mutations were at the same site, resulting in a *MED12*-G44C substitution, consistent with a gain- or change-of-function (Figure 2A). These were mutually exclusive to tumors with a *TERT* promoter mutation (p=0.04) and were also mutually exclusive to *BRAF* (p=0.011) (Figure 2B). The overall survival of patients with *MED12* mutations were similar to those with wild type *MED12* (Figure 2C).

Mutations of the gene *RBM10* were seen in 11% of patients (Figure 2D). *RBM10* mutations were of 2 types: 4 truncating mutations and 2 missense mutations. These were mutually exclusive to tumors with a *BRAF* mutation (p=0.037) but showed co-occurrence with *NRAS* (p=0.027) and *PIK3CA* (p=0.001) (Figure 2E). The overall survival of patients with *RBM10* mutations was significantly poorer to those with wild type *RBM10* (p=0.01, Figure 2F).

TP53, ATM, RB1, POLE mutations—We found mutations in *TP53* (9%), *ATM* (7%) and *RB1* (1.8%) at a higher prevalence than for PTCs in the TCGA (2) (Figure S2A). Mutations in the *POLE* gene were seen in 4/57 (7%) of patients.

PI3K/AKT/mTOR pathway alterations—Mutations of genes encoding members of the *PI3K/AKT/mTOR* pathway were seen in 2557 (44%) of patients. Mutations occurred in *PIK3CA* (4%), *PTEN* (1.8%), *PIK3C2G* (5%), *PIK3CD* (1.8%), *PIK3CG* (1.8%), *PIK3R2* (1.8%), *AKT3* (1.8%), *TSC2* (1.8%) and *RPS6KA4* (1.8%). These mutations tend to be mutually exclusive to one another. (Figure S2B).

Epigenetic gene alterations—Genes encoding components of the SWI/SNF chromatin remodeling complex were mutated in 9/57 (16%) of patients (Figure S2C). Mutations in *ARID1B* (9%), *ARID2* (7%), *SMARCB1* (4%) and *PBRM1* (1.8%) genes were identified. These mutations tended to be mutually exclusive indicating that alteration in only 1 of these genes is sufficient to alter function. It was reported previously that individual SWI/SNF chromatin remodeling complexes can contain e.g. either ARID1A or ARID1B but not both. Namely, the combined absence of ARID1A and ARID1B destabilizes SWI/SNF complexes and results in dissociation of subunits which eventually leads to synthetic lethality (11).

Mutations of the histone methyltransferases (HMTs) *KMT2A* (1.8%), *KMT2C* (4%- 1 amplification, 1 mutation), *KMT2D* (4%), *KDM6A* (1.8%) and *PRDM1* (7%) (1 mutation and 3 amplifications) were found in 10/57 (18%) of tumors (Figure S2C). Additional mutations in other chromatin remodeling and epigenetic regulators were also seen, including histone acetyltransferase *CREBBP* (1.8%) and *BCOR* (4%).

Other gene alterations—Other genes were mutated in a small number of patients. Mutations of other receptor tyrosine kinases (RTKs) such as *PDGFRA* (1.8%), *PDGFRB* (1.8%), *FGFR3* (4%), *ERBB3* (1.8%) and *MET* (1.8%) were identified (Figure S2D). Mutations in *NOTCH2* (5%) and *NOTCH3* (1.8%) occurred in 4/57 (7%) of patients. There were infrequent mutations in *FLT3* (*VEGFR3*) (1.8%), *GNAQ* (1.8%), *GNAS* (4%), *KDR* (1.8%), *ASXL1* (1.8%), *DNMT1* (1.8%), *DNMT3A* (4%).

Gene Fusions (Figure 1)

Of the 57 patients, 4 had a gene fusion identified; 1 in a patient with PDTC and 3 in patients with WDTC.

DLG5-RET—(*DLG5:* 10q23;*RET:* 10q11.2) was identified in 1 WDTC patient with classical papillary thyroid cancer. This is a balanced rearrangement involving *DLG5* exons 1–13, including the N terminal coiled-coil domains, and *RET* exons 12 and the rest of the downstream exons which involves the entire *RET* kinase domain. This patient had no other mutations.

STRN-ALK—(*STRN*: 2p22.2;*ALK*: 2p23) was identified in 1 WDTC (follicular variant of PTC) and 1 PDTC patient. This is an 8 Mb deletion between *STRN* and *ALK* leading to a fusion between *STRN* exons 1–3 and *ALK* exons 20–29 which involves the entire *ALK* kinase domain. One patient also had a *TERT* promoter mutation and the other patient had a *MED12* mutation.

OSBPL1A-BRAF—(*OSBPL1A*: 18q11.1 ;*BRAF*: 7q34) was identified in 1 WDTC patient with tall cell variant of papillary thyroid cancer. This is a balanced rearrangement t(7;18) (q34;q18) involving *OSBPL1A* exons 1–16 and *BRAF* exons 10 and the rest of the downstream exons including the entire *BRAF* kinase domain (AA 457–714). This patient also had a *TERT* promoter mutation.

Copy number alterations (Figure 1, Figure 3)

Of the 57 tumors, there were several arm level alterations identified. The most frequent copy number alterations are shown in Figure 3A. Arm level gains were identified in chromosome 1q in 15 (26%) patients. Arm level losses were identified in chromosomes 22 (14%), 13p (12%), 6q (10%), 1p (9%), 2p (7%), 3p (7%), 17p (7%) and 19p (7%). Example of copy number alterations in 1 tumor are shown in Figure 3B; tumor IG_thy_005 has 1q gain, 6q loss, 9p loss, partial 13 loss, 19p loss and 22 loss. The different components of the FACETS plot are: the top figure shows the GC corrected normalized log-ratio of tumor to normal read depths at a set of SNP loci; the second figure is the log odds ratio from cross tabulating the tumor and normal reads into ref and alt alleles for loci that are heterozygous in the germline; the third figure is the total (black) and minor (red) integer copy number assignment for the segments and the final band shows the cellular fractions where dark blue represents 1 with lighter shades representing lower numbers and beige represents no copy number change.

Pathways altered and mutational comparison to ATC and PTC (Figure 1, Figure 4)

Overall, pathways altered in FNAT included the RAS/PI3K/AKT/MTOR pathway in 40% of patients, DNA damage pathway in 26% patients, chromatin modifying pathways in 33% and alterations in RTKs in 18% of patients (Figure 1). Figure 4 shows the most commonly mutated genes in FNAT compared to well differentiated papillary thyroid cancers (TCGA) and ATC. Compared to WDTC reported in the TCGA, the prevalence of mutations of TERT (60% versus 9%), MED12 (14% versus 0%) and RBM10 (11% versus 0.5%), are higher in FNAT indicating the importance of these genes in tumor virulence. Compared to ATC, FNAT showed a similar MAPK alteration profile with similar frequencies of mutations in

BRAF (40%), *NRAS* (25%) and *HRAS* (4%) genes (1). Mutations in thyroid-stimulating hormone receptor gene (*TSHR*) were also comparable to 6% of ATC (1). Mutations in the eukaryotic translation initiation factor 1A, X–linked (*EIF1AX*), occurred in 12% of our FNAT which was comparable to 9% in our report on ATC (1) and higher than the 1% observed in PTCs (2). We have already reported before a significant association between *EIF1AX* and *RAS* mutation, suggesting that this may predict for more aggressive behavior (1). In contrast, mutations of *PI3K/AKT/mTOR* signaling were uncommon, occurring at a frequency more comparable to PTC (2). Mutations in tumor suppressor genes *TP53* and *RB1* were much less common than in ATC. However, mutated *ATM* showed a similar rate of mutation in more aggressive tumors: 1.3% PTC (2) vs. 7% FNAT vs. 9% ATC (1). Mutations of the *POLE* gene were seen in 7% (4/57) of FNAT patients. DNA polymerase epsilon catalytic subunit (*POLE*) gene mutations affect the active site of the exonuclease domain of DNA polymerase. This mutation has been described in familial forms of colorectal adenomas and cancers of the colon, pancreas, ovaries and small intestine (c. 1373A>T) (12) and in familial cutaneous melanoma (c.1041G>T) (13).

FNAT comprose both WDTC and PDTC.. A comparison of fatal WDTC to nonfatal WDTC using the TCGA cohort is shown in Figure S3A. From this we can conclude that the prevalence of mutations of TERT (60% versus 9%), MED12 (13% versus 0%), RBM10 (4% versus 0.5%) and PIK3CA (4% versus 0.5%) are higher in fatal forms of WDTC. We have also shown the comparison of fatal PDTC to nonfatal PDTC using nonfatal PDTC identified from the cohort in Landa et al (1). This is shown in Figure S3B. From this we can conclude that the prevalence of mutations of TERT (60% versus 21%), MED12 (15% versus 0%), RBM10 (12% versus 0%)) are also higher in fatal forms of PDTC. In addition, prevalence of other genes is higher in fatal forms of PDTC. These included BRAF(29% versus 4%), HRAS (6% versus 1.8%),TP53(15% versus 9%), ATM (12% versus 0%) and EIF1AX(21% versus 4%).

Molecular profile of FNAT carcinomas according to their various histotypes

The molecular alterations categorized by histology are shown in Figure 5. Of the 13 patients with tall cell variant or tall cell features, 9 had both a *TERT* promoter mutations and *BRAF* mutation indicating the importance of this combination in this histology. Of 2 patients with PTC follicular variant, 1 patient had a *TERT* promoter mutation with *NRAS* mutation and the other patient had a *MED12* mutation alone. Of 2 patients with PTC classical type, 1 patient had a *TERT* promoter mutation with a *BRAF* mutation and the other patient harbored the *DLG5-RET* fusion gene. There was 1 patient with a PTC micro carcinoma and this patient had a *MED12* mutation. There were 4 patients with Hurthle cell cancer; 1 patient had *TERT, MED12, RBM10* and *PIK3CA* mutations, 1 patient had *TERT* and *ARID2* mutations, 1 patient had *NRAS* mutation and 1 patient had *ARID1B,POLE, TSHR, FGFR3, TSC2, PDGFRB* and *ERBB3* mutations.

Of patients with PDTC, 60% had a *TERT* promoter mutation either with a *BRAF* mutation or a *NRAS* mutation. 5 patients with *TERT/NRAS* mutations also harbored an *EIF1AX* mutation. All *EIF1AX* mutations occurred in the PDTC patients usually in combination with

NRAS mutations. Of the patients who did not have a *TERT* promoter mutation, mutations were observed in *MED12, RBM10, TP53, and ATM* amongst others.

Discussion

In this study we report a mutational assessment and clinicopathological features of 57 fatal cases of non-anaplastic thyroid (FNAT) cancer and we examine our results in the context of PTC TCGA study (2) and in the context of deep sequencing studies of ATC we reported previously (1).

Telomerase reverse transcriptase (*TERT*) promoter mutations showed the highest prevalence of mutation occurring in 60% of FNAT patients. These occurred with equivalent frequencies in both PDTC (60%) and WDTC (60%) patients. Promoter mutations occurred in the two usual hotspot positions (14). The 60% mutation rate in FNAT is far higher than the 9% of PTCs from TCGA (2) and comparable to 73% of ATC (1). This stepwise increase in the frequency of *TERT* promoter mutations as thyroid differentiation decreases is consistent with our previous reports (1,15) and reports from other studies (16). *TERT* mutations co-occurred with *BRAF* or *RAS*, which enhance the negative prognostic impact of *TERT* promoter mutations (17). In contrast, *TERT* promoter mutations in FNAT were mutually exclusive with *TP53* mutations ($p=0.08$) and with *MED12* mutations ($p=0.04$), consistent with alternate pathways toward progression to FNAT.

Compared to ATC, FNAT also showed a similar MAPK alteration profile (*NRAS, HRAS, BRAF*) and a similar incidence of mutations in the eukaryotic translation initiation factor 1A, X–linked (*EIF1AX*) (18). In contrast, mutations of *PI3K/AKT/mTOR* signaling were uncommon, occurring at a frequency more comparable to PTC (2). In addition, mutations in tumor suppressor genes *TP53* and *RB1* were much less common than in ATC, supporting the notion that mutations in *TP53* are infrequent in all histologic types of thyroid cancer with the exception of ATC (1).

We identified chromosomal rearrangements in only a small percentage of FNAT (4/57; 7%) and all involved the entire kinase domain of the fusion partner. Importantly, 2 of the 3 fusion genes identified have never been reported before (*DLG5-RET* and *OSBPL1A-BRAF*). *DLG5-RET* fusion involved the entire *RET* kinase domain and therefore *DLG5* may lead to constitutive activation of *RET* kinase. Disc large homolog 5 (*DLG5*) gene is located in a region that undergoes substantial recombination and has a possible role in inflammatory bowel and Crohns disease (19), in cell division, proliferation, cell migration and invasion (20), however it has not been reported as a *RET* partner in chromosomal rearrangements. As with other *RET* fusion partners, the *DLG5* gene has a coiled-coil domain which acts as the dimerisation domain for the *RET* gene. *OSBPL1A-BRAF* is a balanced rearrangement that involves the entire *BRAF* kinase domain. In this novel fusion, *OSBPL1A* may lead to constitutive activation of *BRAF* kinase. *OSBPL1A* (Oxysterol-binding protein-related protein 1, which acts as an intracellular lipid receptor which is a member of the oxysterol-binding protein family) was shown to have differential expression of isoforms in several cancer types as a result of alternative transcription start site (in colorectal, lung, bladder, liver, prostate, gastric, and brain cancer) (21). The other fusion identified was *STRN-ALK.*

This involves the entire *ALK* kinase domain, which leads to constitutive activation of *ALK* kinase via dimerization mediated by the coiled-coil domain of *STRN* (22). This rare rearrangement has been reported in thyroid cancer before (2) as well as renal cell carcinoma (23) and colorectal adenocarcinoma (24). Patients with this fusion have shown significant initial clinical response to the ALK inhibitors crizotinib and TAE864.

Several copy number alterations (CNA) were identified with the most common being gain of chromosome 1q and also loss of chromosome 22. Chromosome 1q gain was present in 15 FNAT patients (26%), which is higher than 14.8% (2) and 16% (25) reported in PTC. In PTC, 1q gain has been reported in more aggressive tumors (26) and associated with significantly higher MACIS scores, risk profiles and PTC tumor stage (2) as well as distant metastases (25). In PDTC, 1q gains were among the most common arm level CNA (1) and PDTC patients with 1q gains had worse survival rates (1). The high incidence of 1q gain that we observe is in keeping with these findings. Arm level losses in chromosome 22 were present in 8 FNAT patients (14%). This corresponds with previous reports on PTC and PDTC where 22q loss was reported (1,2,25). Loss of 22q region includes tumor suppressor genes *NF2* and *CHEK2* (25). *NF2* loss promotes *RAS* induced tumorigenesis (27) which is consistent with strong association between 22q loss and *RAS*-mutated PDTC (1). Therefore our report of 1q gain and 22 loss in FNAT is consistent with previous reports of these CNA in aggressive thyroid tumors.

Our study identified a remarkably high prevalence of mutations in 2 genes, *MED12* and *RBM10*, suggesting a role of these genes in tumor virulence. When we carried out a comparison of fatal forms of WDTC and PDTC to nonfatal forms of WDTC and PDTC, these 2 genes had a higher mutation prevalence indicating their importance in both WDTC and PDTC tumor virulence. *MED12* (Mediator of RNA polymerase II transcription subunit 12 homolog) is located on X chromosome and encodes for a subunit of the macromolecular complex known as Mediator. Mediator complex consists of the core Mediator and the kinase module and initiates DNA transcription by interacting with RNA polymerase II (RNA Pol II) (28). Since *MED12* plays an essential role in the assembly and activation of the kinase module (29,30) mutations in *MED12* can lead to loss or gain of kinase activity. The latter can act as a promoter or suppressor of tumorigenesis, depending on biologic function that the kinase module carries out in the particular tissue (31,32).

MED12 has recently been included as a cancer driver gene in a recent large scale genomic analyses (33,34), reflecting its growing importance. In our study of FNAT, *MED12* mutation clustered in a hotspot region in the N terminal region of exon 2 and this clustering suggests a specific change of function. This is consistent with the vast majority of reports where missense mutations of *MED12* clustered in a hotspot region within exon 2 (31). Mutation of *MED12* has been reported to alter highly conserved amino acids residues (L36, Q43 and G44) in exon 2 (35) which points to a possible gain or change of function. Exon 2 mutations were initially found in uterine leiomyomas (UL) (35) and were the first *MED12* mutations reported in human tumors which implicated the role for disrupted Mediator-associated CDK8 kinase activity in tumorigenesis. Since then, *MED12* mutations have been reported in typical UL (up to 86%), breast fibroadenomas (59–67 %) and phyllodes tumors (80–88%) (31). Comparative expression profiling and gene set enrichment analyses from mutant and

wt *MED12* reveal TGF-β signaling and Wnt/β-catenin signaling in mutant UL (31). Further research is needed to determine the impact of *MED12* exon 2 mutations on the estrogen signaling pathway and its possible dysregulation during tumorigenesis. Exon 2 mutations are recurrent albeit less frequent in malignant uterine leiomyosarcomas (4–30%), chronic lymphocytic leukemias (5%) and colorectal cancers (0.5%) (31). *MED12* mutations have also been reported outside of exon 2 in 5% of prostate cancers and may act through disruption of CDK8 kinase with subsequent transcriptional dysregulation of p53 and androgen signaling (36,37). In our study, all *MED12* mutations were recurrent mutation in a single codon resulting in a MED12-G44C substitution. We anticipate that the *MED12* mutations in our patients may represent gain or change of function. However, given the complexity of the Mediator complex and the variability in either gain or loss of function depending on tumor type, more research is required to properly explore the true function of the *MED12* mutation that we have identified.

In addition to *MED12* mutations, we also found mutations in the RNA Binding Motif Protein 10 (*RBM10*) in 11% of FNAT patients. *RBM10* is an RNA binding protein which participates in alternative pre-mRNA splicing. Splicing has a direct role in regulation of gene expression and maintaining the homeostasis of cellular processes. Indeed there has been growing evidence of the involvement of mutated splicing factors in tumor progression (38). Mutation of splicing factors can impair expression of genes crucial for maintaining homeostasis of cell growth and therefore represents a novel mechanism which may promote growth advantage and tumorigenesis of select clonal populations. Mutations of genes encoding splicing factors have been most commonly reported in hematologic malignancies (myelodysplastic syndromes (MDS), acute myeloid leukemia, and chronic lymphocytic leukemia), and less frequently in several solid tumors (38). Most frequent mutations occur in *SF3B1, U2AF1, SRSF2* and *ZRSR2* and are generally mutually exclusive (38). With regards to *RBM10*, mutations have been reported in lung adenocarcinomas (39,40), where it acts as an alternative splicing regulator (41) modulating the product of *NUMB*, a NOTCH pathway regulator gene (42) critical for progression of lung adenocarcinomas. Studies point to loss of tumor suppressor properties of wild type *RBM10* and oncogenic function of mutated *RBM10* as possible mechanisms for causing uncontrolled growth (40). *RBM10* knockdown (*RBM10KD*) in human cancer cells enhanced tumor growth of xenografts in nude mice with similar results in lung adenocarcinoma cells expressing an RBM10 valine to glutamic acid (V354E) substitution (40). In addition to missense mutations, truncation mutations also occur in *RBM10*. RBM10 truncated mutants lacking the C-terminal Zn-finger and glycin patch are basically non-functional; moreover the shortest variants appear to exert a dominant-negative effect (40). In our study, *RBM10* mutations were of 2 types: 4 truncating mutations and 2 missense mutations. We anticipate that the *RBM10* mutations in our patients may represent loss of tumor suppressor function. Furthermore, our study showed statistically significant co occurrence of mutation in *RBM10* with *NRAS* and *PI3KCA* and also a mutual exclusivity with *BRAF* mutations. This suggests mutual independence in oncogenic potential of *RBM10* regulated proteins and BRAF signaling. Importantly, patients who had *RBM10* mutations had a significantly poorer survival compared to patients who did not have these mutations. This suggests tumors harboring *RBM10* mutations are biologically more virulent.

Our study has identified several genetic alterations which may have therapeutic implication. There is a great interest to specifically target mutated *TERT* promoter and our findings suggest aggressive thyroid cancer with these mutations would be an ideal cancer to treat. In addition, new insights into *TERT* genetics and biology may also offer a potential for personalized immunotherapy (43). The rare *STRN-ALK* rearrangement can be targeted with ALK inhibitors, crizotinib and TAE864, as previously mentioned. The central role of *MED12* in the proper function & assembly of the Mediator kinase module makes MED12 an attractive therapeutic target. So far efforts in targeted therapy have mostly been directed towards CDK8 kinase; e.g. Sorafenib as a multi tyrosine kinase inhibitor/CDK8 inhibitor and Senexin A as a novel CDK8/19 inhibitor (31). An important obstacle in targeting Mediator kinase module may be its versatile role in both repression and activation of transcription depending on the context (31,44) and consequent impact on oncogenic or tumor suppressor signaling. Mutated *RBM10* may also represent a novel therapeutic strategy at the level of transcription. Splicing factor inhibitors have already been tested in clinical trials but not in patients with splicing factor mutations (38). A phase I trial of E7107, a spliceosome inhibitor, have been conducted in patients with advanced solid tumors unresponsive to standard therapies (45) and showed promising results. It is possible that splicing factor inhibitors may also be useful in patients which harbor *RBM10* mutations.

In conclusion we report the mutational profile of the largest series of fatal NAT that has not been reported before. We have identified *TERT* promoter mutations in a very high percentage of tumors indicating its importance in thyroid cancer virulence. We report a high incidence of chromosome 1q gain which highlights its importance in tumor aggressiveness. We have identified 2 novel fusion genes *DLG5-RET and OSBPL1A-BRAF*. Lastly, we report on many novel genes not previously reported in differentiated thyroid cancer including *MED12* and *RBM10* suggesting a role for these novel genes in tumor virulence. This new data will clarify the genetic basis of the most virulent forms of thyroid cancer and will therefore help focus future therapeutic directions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of translational relevance

We report the mutational profile of the largest series of fatal non anaplastic thyroid cancer that has not been reported before. We have identified *TERT* promoter mutations in a very high percentage of tumors indicating its importance in thyroid cancer virulence. We report a high incidence of chromosome 1q gain which highlights its importance in tumor aggressiveness. We have identified 2 novel fusion genes *DLG5-RET and OSBPL1A-BRAF*. Lastly, we report on many novel genes not previously reported in differentiated thyroid cancer including *MED12* and *RBM10* suggesting a role for these novel genes in tumor virulence. These genes are targetable mutations and therefore have translational importance in thyroid cancer management. The study also has diagnostic importance as these genetic alterations may predict for poor outcome in patients presenting with thyroid cancer allowing the early identification of patients who may benefit from more aggressive therapy.
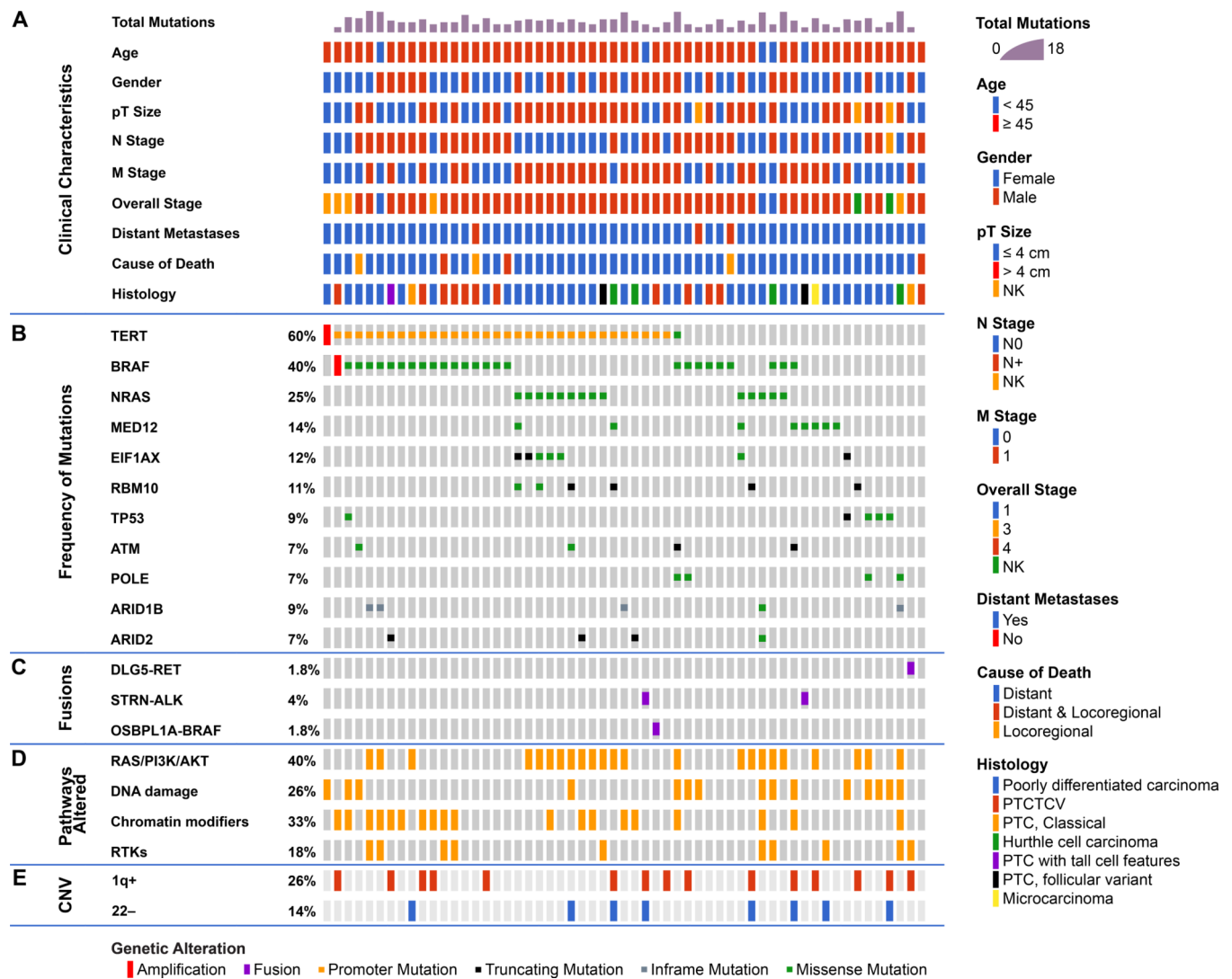
Figure 1.
Genomic landscape of fatal non-anaplastic thyroid cancer found in 410 genes in MSKCC IMPACT. Clinicopathological characteristics (A) included age, gender, tumor size, N stage, M stage, overall stage, distant metastases and histology. The most frequent genes mutated are shown in panel (B) with fusions shown in panel (C). The percentage of tumors with alterations in different pathways is shown in panel (D); RAS/PI3K/AKT pathway includes NRAS, HRAS, PIK3CA, ,PTEN, PIK3C2G, PIK3CG, AKT1, AKT3, TSC2, and MTOR),Chromatin modifier genes including KMT2A, KMT2C, KMT2D, KDM6A, PRDM1, BCOR, NCOA3, HIST1H3H, HIST1H1C, ARID1B, ARID2, SMARCB1, PBRM1, ATRX, CREBBP. Alterations in DNA damage control included mutations in TP53, RB1, MSK2, CHEK2, and POLE. Alterations in RTKs included mutations in PDGFRA, PDGFRB, FGFR3, ERBB3, MET, EGFR, TSHR, FGF3, TGFBR1, IFNGR1. The percentage of tumors with gain of chromosome 1q and loss of chromosome 22 are shown in panel (E).
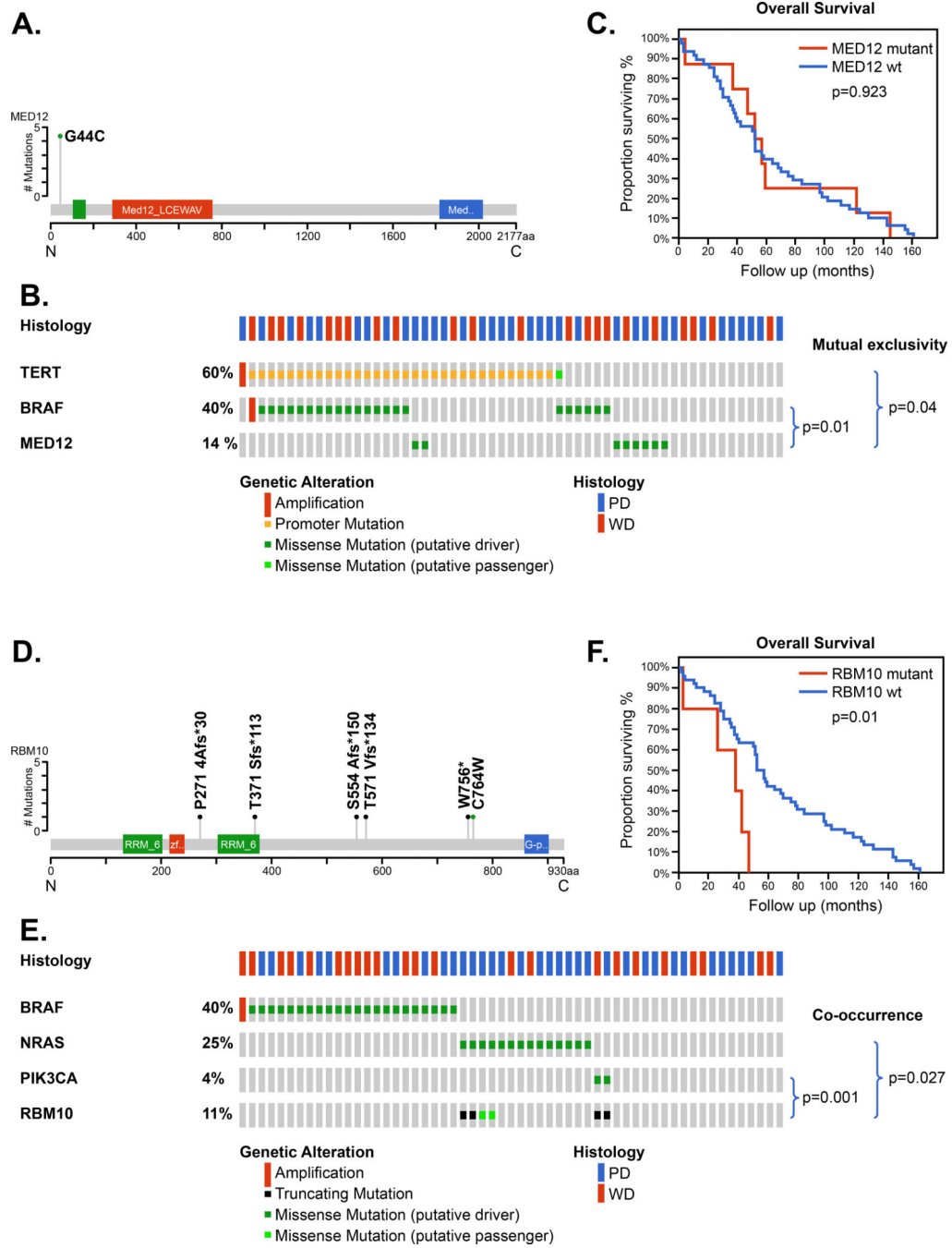
Figure 2.
Mutations in MED12 and RBM10 in fatal non-anaplastic thyroid cancer
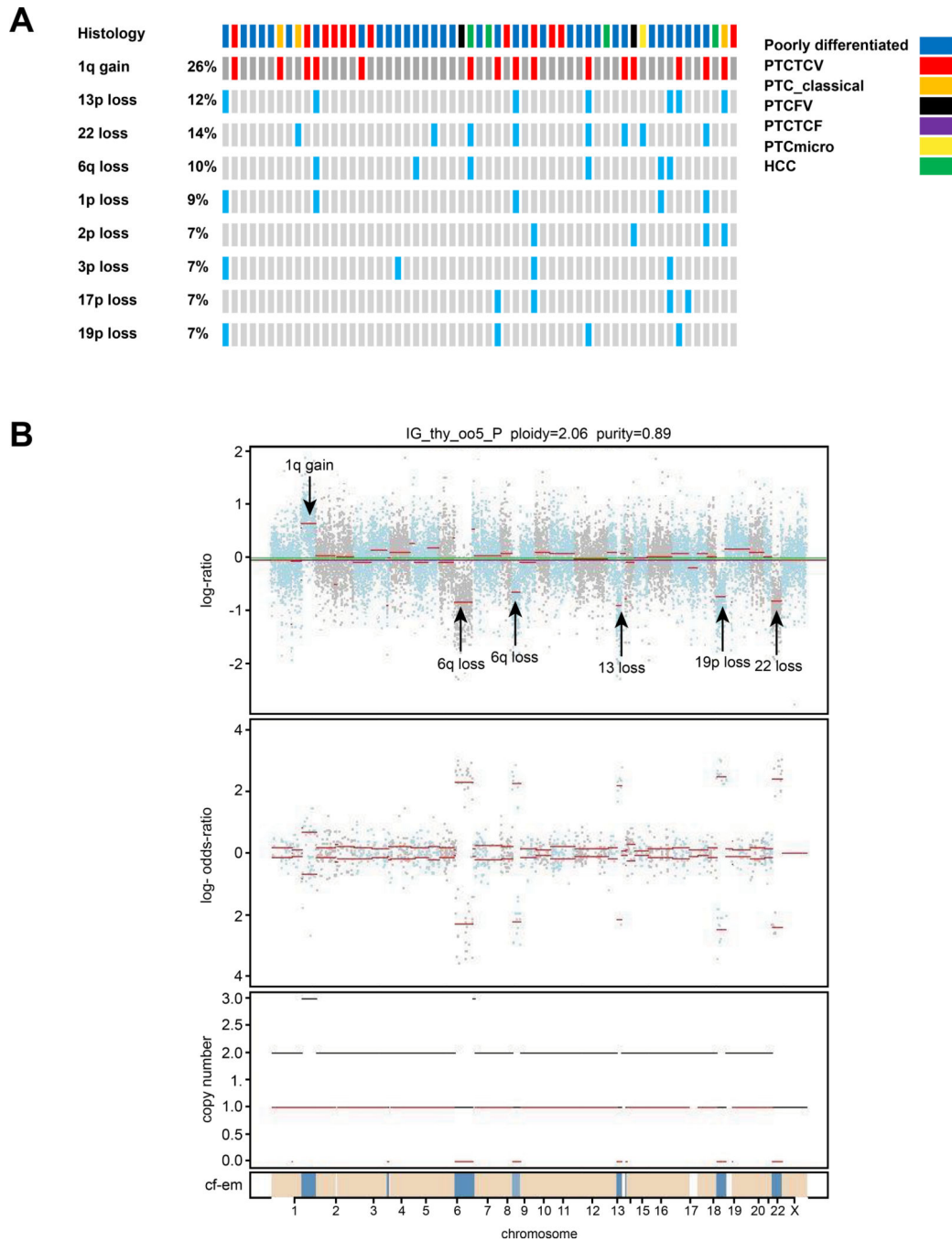
Figure 3.
Most frequent copy number alterations for each patient are shown in A. Figure 3B shows an example of copy number alterations using FACETS in tumor IG_thy_005 which has 1q gain, 6q loss, 9p loss, partial 13 loss, 19p loss and 22 loss.
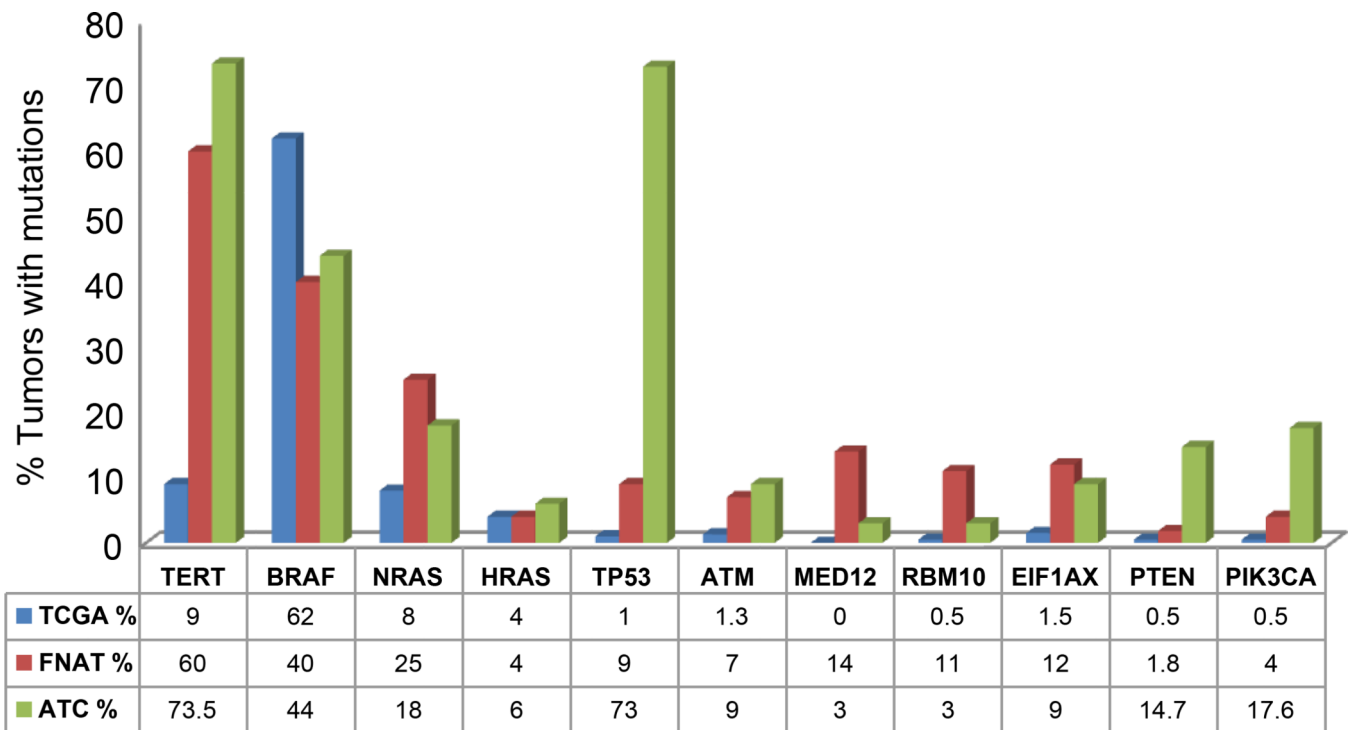
	TERT	BRAF	NRAS	HRAS	TP53	ATM	MED12	RBM10	EIF1AX	PTEN	PIK3CA
■ TCGA %	9	62	8	4	1	1.3	0	0.5	1.5	0.5	0.5
■ FNAT %	60	40	25	4	9	7	14	11	12	1.8	4
■ ATC %	73.5	44	18	6	73	9	3	3	9	14.7	17.6

Figure 4.
Comparison of the most commonly mutated genes in well differentiated thyroid cancer
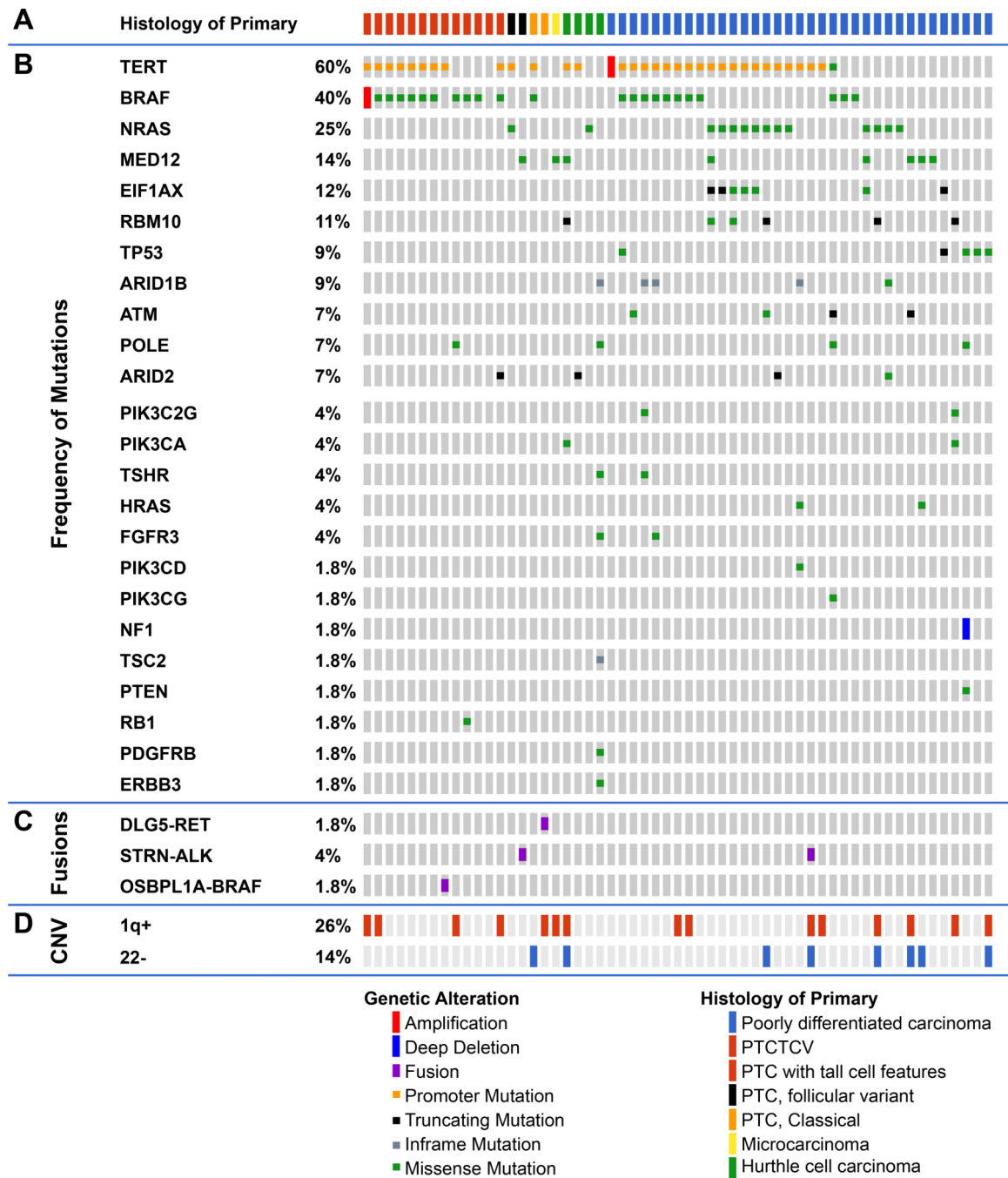(TCGA), fatal cases of nonanaplastic thyroid cancer and anaplastic thyroid cancer.

Figure 5.
Molecular alterations in FNAT stratified by histology

Table 1

Patient, tumor, treatment and outcome characteristics of 57 patients with fatal non anaplastic thyroid cancer

		N	%
Age	< 45 years	5	9%
	45 years	52	91%
Sex	Female	32	56%
	Male	25	44%
pT size	4 cm	22	39%
	> 4 cm	32	56%
	Unknown	3	5%
pT stage	T1	2	4%
	T2	0	0%
	T3	19	32%
	T4	34	60%
	TX	2	4%
ETE	No	9	15%
	Yes	46	81%
	microscopic	12	21%
	gross	34	60%
	Unknown	2	4%
Margins	Negative	25	44%
	Positive/Close	30	52%
	Unknown	2	4%
pN stage	N0/Nx	25	44%
	N1a	8	14%
	N1b	23	40%
	Unknown	1	2%
M stage	M0	27	47%
	M1	30	53%
Stage	1	3	5%
	2	0	0%
	3	5	9%
	4	47	82%
	Unknown	2	4%
Tm grade	PDTC	35	61%
	WDTC	22	39%

ETE extrathyroid extension

pN_X clinically negative neck