Limitations of Detecting Genetic Variants from the RNA Sequencing Data in Tissue and Fine-Needle Aspiration Samples

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Background: Genetic profiling of resected tumor or biopsy samples is increasingly used for cancer diagnosis and therapy selection for thyroid and other cancer types. Although mutations occur in cell DNA and are typically detected using DNA sequencing, recent attempts focused on detecting pathogenic variants from RNA. The aim of this study was to determine the completeness of capturing mutations using RNA sequencing (RNA-Seq) in thyroid tissue and fine-needle aspiration (FNA) samples.

Methods: To compare the detection rate of mutations between DNA sequencing and RNA-Seq, 35 tissue samples were analyzed in parallel by whole-exome DNA sequencing (WES) and whole-transcriptome RNA-Seq at two study sites. Then, DNA and RNA from 44 thyroid FNA samples and 47 tissue samples were studied using both targeted DNA sequencing and RNA-Seq.

Results: Of 162 genetic variants identified by WES of DNA in 35 tissue samples, 77 (48%) were captured by RNA-Seq, with a detection rate of 49% at site 1 and 46% at site 2 and no difference between thyroid and nonthyroid samples. Targeted DNA sequencing of 91 thyroid tissue and FNA samples detected 118 pathogenic variants, of which 57 (48%) were identified by RNA-Seq. For DNA variants present at >10% allelic frequency (AF), the detection rate of RNA-Seq was 62%, and for those at low (5–10%) AF, the detection rate of RNA-Seq was 7% (p < 0.0001). For common oncogenes (BRAF and RAS), 94% of mutations present at >10% AF and 11% of mutations present at 5–10% AF were captured by RNA-Seq. As expected, none of TERT promoter mutations were identified by RNA-Seq. The rate of mutation detection by RNA-Seq was lower in FNA samples than in tissue samples (32% vs. 49%, p = 0.02).

Conclusions: In this study, RNA-Seq analysis detected only 46–49% of pathogenic variants identifiable by sequencing of tumor DNA. Detection of mutations by RNA-Seq was more successful for mutations present at a high allelic frequency. Mutations were more often missed by RNA-Seq when present at low frequency or when tested on FNA samples. All TERT mutations were missed by RNA-Seq. These data suggest that RNA-Seq does not detect a significant proportion of clinically relevant mutations and should be used with caution in clinical practice for detecting DNA mutations.

Keywords: thyroid FNA, mutations, targeted NGS, RNA-Seq

Introduction

Genetic profiling of human tumors is increasingly used to improve cancer diagnosis and prognostication and to identify potential therapeutic targets (1). For thyroid nodules, molecular testing is frequently used when fine-needle aspiration (FNA) cytology is indeterminate, and more accurate prediction of cancer probability is needed to inform patient management (2). A number of genetic alterations, such as TERT promoter mutations, have emerged as

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important prognostic markers that define the most aggressive class of thyroid cancers (3). In advanced thyroid cancer, genetic analysis is helpful to identify therapeutic targets such as *BRAF*<sup>V600E</sup> and *RET* mutations and *NTRK* and *ALK* gene fusions (4).

Although sequencing of DNA isolated from cells collected by FNA or tumor sections is a standard approach used for detecting DNA variants, the utility of RNA converted to cDNA has been recently explored as an alternative template for sequencing and detection of DNA mutations (5–9). Such approach is based on the premise that specific DNA regions encoding genes that are expressed in the tissue of interest are transcribed into mRNA, and DNA mutations located in the coding regions of these genes can be detected by RNA sequencing (RNA-Seq).

The use of RNA for detecting DNA variants would simplify the workflow as it would allow detection of two main classes of genetic alterations found in thyroid tumors, that is, point mutations and gene fusions, using a single RNA-based approach. However, the use of RNA for detecting gene mutations is expected to be limited to the expressed gene areas and depends on the stability and abundance of mRNA transcribed from the gene of interest in specific cell types. Indeed, several studies have demonstrated that only about half of all genetic variants detectable by sequencing of human tissue and cell line DNA could be captured using RNA-Seq (5–8). Furthermore, only limited information is available on the completeness of detection of genetic variants using the RNA-Seq data in thyroid nodules and cancer (9).

The aim of this study was to assess the completeness of capturing DNA mutations using RNA-Seq. To achieve this aim, we performed side-by-side sequencing of DNA and RNA from tissue and FNA samples and compared the detection rate of mutations in different genes and DNA regions using these two sequencing approaches.

**Materials and Methods**

**Samples and study design**

De-identified DNA and RNA samples isolated from 35 randomly selected tissue specimens were studied using whole-exome DNA sequencing (WES) and whole-transcriptome (RNA-Seq) analysis at two study sites, the Englander Institute for Precision Medicine, Weill Cornell Medicine, New York, NY (site 1, *n* = 18, brain tumors) and the University of Pittsburgh Medical Center, Pittsburgh, PA (site 2, *n* = 17, thyroid tumors) with the approval by respective institutional review boards. In addition, DNA and RNA from 44 thyroid FNA samples and 47 thyroid tissues were studied by targeted DNA sequencing (112-gene panel) and RNA-Seq at the University of Pittsburgh Medical Center. The samples were pre-selected to represent all mutations most commonly occurring in thyroid cancer and mutations present at various levels including low level. The summary of the study design is given in Figure 1. Detected genetic alterations are summarized in Supplementary Tables S1–S3.

**Whole-exome DNA sequencing**

Whole-exome DNA sequencing was performed using DNA as a template independently at the two study sites. At site 1, DNA was isolated and whole-exome DNA sequencing was performed as previously reported (10). Briefly, library preparations were performed using Agilent HaloPlex Library (Agilent Technologies, Inc., Santa Clara, CA). Sequencing was conducted on Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA). Sequencing reads alignment was performed using BWA with GRC37/hg19 reference genome (11), and mutations were detected using EXaCT-1 pipeline v0.9 using patient-matched tumor/normal samples (10).

At site 2, DNA isolation and whole-exome DNA sequencing were carried out as previously described (12). Libraries were prepared using KAPA HyperPlus and xGen Exome Research Panel v1.0 (Integrated DNA Technologies, Inc., Coralville, IA). Sequencing was performed on Illumina NovaSeq using NovaSeq S1 300 Kit (Illumina) and reads alignment using BWA aligner (11) and Picard tools. For all analyses, the reference genome build GRC37/h19 was used. The aligned reads were preprocessed using Genome Analysis Tool Kit (GATK) (13). Mutations were detected using MuTect2 pipeline using “somatic only” settings. A panel of normal variants was created using data from the 1000 Genomes Project (14). The variants were annotated using ANNOVAR and filtered using quality metrics such as variant quality score recalibration, in-house database, and external databases such as Cosmic (15,16).

**Whole-transcriptome sequencing (RNA-Seq)**

RNA-Seq analysis and detection of mutations were performed using protocols established at each study site. At site 1, RNA isolated from frozen specimens was prepared for RNA-Seq using TruSeq RNA Library Preparation Kit v2 or riboZero as previously described (17). Sequencing was performed on GAI, HiSeq 2000, or HiSeq 2500. RNA-Seq and data processing were conducted as previously reported (18,19). The reads were aligned using STAR_2.4.0f1 (20). SAMTOOLS v0.1.19 was used for sorting and indexing reads (21). Cufflinks (2.0.2) was used to estimate the expression values (FPKM) (22) and GENCODE v19 GTF file for annotation (23). Since the sequenced samples from the published data sets were processed using different library preps, batch normalization was performed using ComBat (24) from bioconductor package (25). Mutations were identified using in-house program maseqnut (v0.6).

At site 2, RNA isolation and sequencing were performed as previously reported (12). To detect mutations from whole-transcriptome data, the best practice guidelines for RNA-Seq short variant discovery from the Broad Institute were used. The preprocessing was performed on raw RNA-Seq reads.

**FIG. 1.** Summary of the study design.
Targeted next generation sequencing (NGS) panel

Targeted next generation sequencing analysis was performed to genotype surgically removed tissue samples \((n = 47)\) and FNA samples \((n = 44)\) from thyroid nodules using the ThyroSeq v3 GC assay, as previously described \((27)\). The assay uses targeted amplification-based NGS technology to detect genomic alterations in 112 thyroid-related genes by sequencing DNA and RNA on the Ion GenoStudio SS System \((\text{Thermo Fisher Scientific, Waltham, MA})\) according to the manufacturer’s protocol. To detect mutations from targeted NGS data, the signal data from sequencing were analyzed using Torrent Suite software v5.8. For 17 thyroid tumor samples that were sequenced by whole-exome DNA sequencing, targeted DNA sequencing, and RNA-Seq, all discrepant mutations were visually inspected in addition to automated variant calling pipeline.

Statistical analysis

Statistical analysis was performed using RStudio \((1.0.136)\) package with R \((v3.3.2)\) and ggplot2 \((2.2.1)\). Comparison between the detection rate of DNA variants by different sequencing approaches was performed using \(t\)-test; \(p\)-values were two-sided and considered significant if \(< 0.05\). To evaluate the accuracy of detecting pathogenic variants by several approaches, positive percentage agreement (PPA) was calculated \((28)\). To compare the differences in the allelic frequency (AF), root mean square (RMS) error was used. All confidence intervals were two-sided 95% and were computed using the Wald test \((29)\).

Results

Detection of genetic variants by RNA-Seq compared with whole-exome DNA sequencing

To evaluate the rate of detection of cancer-related genetic variants that occur in DNA using tumor RNA, 35 tumor tissue samples were analyzed using whole-exome DNA sequencing and whole-transcriptome RNA-Seq at two participating study sites. Each study site performed detection of variants independently using their own clinical sequencing protocols and bioinformatics pipelines. Only cancer-related genetic variants were used for comparison between sequencing of DNA

| Gene/variants | Variants detected by targeted DNA sequencing | Variants detected by RNA-Seq | RNA-Seq (PPA\(^a\)) |
|---------------|---------------------------------------------|-----------------------------|----------------------|
| **BRAF**      | 27                                          | 13                          | 48% (29–67%)         |
| **RAS (NRAS, HRAS, KRAS)** | 25                                          | 19                          | 76% (59–93%)         |
| **TERT**      | 23                                          | 0                           | 0%                   |
| **TP53**      | 16                                          | 12                          | 75% (54–96%)         |
| **E1F1AX**    | 15                                          | 6                           | 40% (15–65%)         |
| **PTEN**      | 6                                           | 3                           | 50% (10–90%)         |
| **PIK3CA**    | 3                                           | 1                           | 33% (0–87%)          |
| **TSHR**      | 2                                           | 2                           | 100%                 |
| **Dicer1**    | 1                                           | 1                           | 100%                 |
| All variants  | 118                                         | 57                          | 48% (39–57%)         |

\(^a\)PPA is accuracy of detection of cancer-related genomic variants by RNA-Seq, which is calculated as a percentage of variants detected by RNA-Seq out of all genomic variants detected by DNA sequencing (WES). PPA, positive percentage agreement; RNA-Seq, RNA sequencing; WES, whole-exome DNA sequencing.

Detection rate of mutations varied for different genes. We further analyzed the sequencing data to examine whether the rate of detection of mutations by RNA-Seq varies...
between different mutations. The most common mutations detected by targeted DNA sequencing in thyroid samples were BRAF (n = 27) and RAS (n = 25), of which RNA-Seq detected 13 (48%) of BRAF and 19 (75%) of RAS mutations (Table 2). For tumor suppressor genes TP53 and PTEN, the detection rate was 75% and 50%, respectively. Other mutations were detected in expressed RNA at various rates, from 33% for PIK3CA and 44% for EIF1AX splice mutations to 100% for TSHR (Table 2). Finally, none of 23 samples with TERT promoter mutations (19 C228T, 4 C250T) were detected using RNA-Seq. If TERT promoter mutations were excluded, the overall detection rate of expressed DNA variants by RNA-Seq would increase from 48% to 68%.

Detection rate dropped for low allelic frequency mutations. Next, we determined whether the rate of detection of mutations in these samples varied for mutations present at different allelic frequencies. Allelic frequency of a mutation is calculated as a percentage of sequencing reads containing the mutation divided by total number of reads covering the locus. It allows estimating the proportion of clonal tumor cells to all (neoplastic and nonneoplastic) cells collected from each sample (by multiplying the allelic frequency by 2 as most mutations are heterozygous). This is important for clinical samples that may contain low proportion of cancer cells, such as thyroid FNA samples. This is because thyroid tumors not infrequently have significant infiltration by lymphocytes and other inflammatory cells, stromal fibroblasts, or other nonneoplastic cells that “dilute” tumor cells collected by FNA.

In our group of samples, among 118 DNA mutations detected by targeted DNA sequencing, 89 had allelic frequency of >10% (i.e., a heterozygous mutation present in >20% of collected cells), which was designated as “high.” The remaining 29 variants had a “low” allelic frequency of 5–10% (i.e., were present in 10–20% of collected cells). The rate of mutations detected by RNA-Seq was 80% for BRAF, 50% for RAS, and 58% for other mutations. For mutations with a 5–10% allelic frequency, the detection rate by RNA-Seq was 80% for BRAF, 50% for RAS, and 58% for other mutations. For mutations present in the FNA samples at an allelic frequency of >10%, the detection rate by RNA-Seq increased to 47% if the TERT promoter mutations are excluded. The detection rate would increase to 47% if the TERT promoter mutations are excluded. The rate of detection of gene variants was significantly lower compared with tissue samples (36% vs. 49%, p = 0.02), which was designated as “low.” The remaining 29 variants had a “low” allelic frequency of 5–10% (i.e., were present in 10–20% of collected cells). When the rate of mutations detected by RNA-Seq was examined separately in different allelic frequency groups, we observed that 62% of high allelic frequency mutations and only 7% of low allelic frequency mutations were captured using RNA (p < 0.0001). These detection rates without TERT promoter mutation in these data sets will be 78% and 8% for “high” and “low” AF, respectively. Among the most common mutations that affect BRAF and RAS oncogenes, 94% of those present at a high (>10%) allelic frequency and 11% of those present at a low (5–10%) allelic frequency were captured by RNA-Seq. None of the TP53, PIK3CA, and EIF1AX mutations present at low allelic frequency were detected using the RNA-Seq. None of the TERT mutations were captured by RNA-Seq regardless of allelic frequency. These results are summarized in Figure 2.

Detection rate of mutations in thyroid FNA samples. When the detection of gene variants was examined separately in the 44 FNA samples, of 74 variants identified using targeted DNA sequencing, 27 (36%) were detected in expressed RNA (Supplementary Table S1). The detection rate would increase to 47% if the TERT promoter mutations are excluded. The difference in the detection rate was due to more common presence of variants with low allelic frequency in the FNA samples (i.e., 38% of thyroid FNA samples had low-level variants compared with 7% of thyroid tumor tissues). Indeed, among mutations present in the FNA samples at an allelic frequency of >10%, the detection rate by RNA-Seq was 80% for BRAF, 50% for RAS, and 58% for other mutations. For mutations present at a low (5–10%) allelic frequency, the detection rate by RNA-Seq dropped to 7% for BRAF and RAS, whereas TP53 and EIF1AX mutations were not detected (Supplementary Table S1).

AF of mutations cannot be reliably detected by RNA-Seq. Finally, we explored the accuracy of detection of allelic frequency of gene mutations in the RNA-Seq data. Ninety-one thyroid tissue and FNA samples were used to compare the mutation allelic frequency detectable using the RNA-Seq data with those calculated using targeted DNA sequencing. Only variants that were detected by both methods were included in the comparison. Overall, correlation between the two methods was poor and showed a RMS error of 0.26 (Fig. 3). These results suggest that AFs of gene mutations may not be reliably determined using the RNA-Seq data.

**FIG. 2.** RNA-Seq detection rate of mutations that were present at high and low allelic frequencies on DNA sequencing: a study of 47 thyroid tissues and 44 thyroid FNA samples sequenced by RNA-Seq and targeted next generation sequencing panel. FNA, fine-needle aspiration; RNA-Seq, RNA sequencing.
mRNA molecules. The low number of copies of mRNA to decreased stability and increased degradation of respective source tissue, and some loss-of-function mutations may lead RNA-Seq data is that not all genes are well expressed in the tended for clinical decision-making.

high-risk cancer (30). Such limitations of RNA-Seq should be taken into account when the results of FNA analysis are in-

RAS and low-risk cancer. In contrast, the correct identification of both promoter mutations are important diagnostic and particularly could be due to several reasons, including difference in mutation profiles between the tested samples. The current study included samples with a broader range of mutated genes, including BRAF, RAS, TP53, PTEN, and TERT, whereas in the study by Angell et al., most of the samples were positive for BRAF, RAS, and TSHR mutations. Notably, even among thyroid samples that did not carry TERT, TP53, and other rare but clinically relevant mutations, 47/181 (26%) of mutations were missed when using RNA-Seq (9).

Similar or higher rates of genetic variants missed by RNA-Seq can be expected in thyroid FNA samples compared with molecules makes the detection of mutations in these genes more difficult by RNA-Seq. In one study, after the analysis was restricted to those genes that were well expressed in the source tissue, the detection rate of genetic variants increased from 40% to 81% (7). In thyroid tissues, this may affect the detection of loss-of-function mutations that occur in tumor suppressor genes, such as TP53 and PTEN. Among those, TP53 mutations are of particular importance as they represent a marker of more invasive, high-risk thyroid cancers, particularly in Hürthle cell tumors (31,32).

The third reason that can affect the detection of DNA variants using RNA-Seq is related to the intrinsic complexity of the computational analysis of RNA data, which require correct mapping of RNA-Seq reads to the reference human genome (6,8). Such analysis has to account for mRNA splicing, which can be affected by different factors including mutations at the gene splice sites. One of such genes relevant to thyroid cancer is EIF1AX, which harbors mutations frequently affecting the splice sites between intron 5 and exon 6 of the gene (33). Indeed, in this study, only 6 of 15 (40%) EIF1AX mutations were captured when using RNA-Seq.

Yet another factor that may affect the detection of heterozygous mutations by RNA-Seq is related to expression balance and specific situations when the normal allelic of the gene is more highly expressed than the mutant allelic (6,34). Deviation from a 1:1 ratio of the mutant:normal allelic present in DNA in favor of the normal allelic in the sequencing reads generated by RNA-Seq can impair the detection of such mutations, particularly when they are present at a low allelic frequency. A significant drop in the detection rate of mutations present at low allelic frequency (5–10%) was observed for most mutations in this study. This confirmed previously reported findings by Angell et al. who observed that the detection rate of genetic variants in thyroid samples analyzed by RNA-Seq was higher for the variants present at an allelic frequency of >20% compared with those present at an allelic frequency of >5% (9). Detecting low-level mutations in thyroid FNA samples is diagnostically important, particularly for mutations that have strong association with cancer, such as $BRAF^{V600E}$. This mutation, even when found at a low allelic frequency (e.g., 5%), confers a very high probability of cancer. Missing low-level $BRAF^{V600E}$ mutation in the FNA sample would decrease diagnostic accuracy of RNA-Seq for nodules with only partial cancer sampling or those with extensive infiltration by lymphocytes or other inflammatory cells, which would more often yield FNA samples containing a small proportion of cells carrying a given mutation.

The difference in the overall detection rate of genetic variants in thyroid samples by RNA-Seq between this study and one reported by Angell et al. (9) (48% vs. 74%, respectively) could be due to several reasons, including difference in mutation profiles between the tested samples. The current study included samples with a broader range of mutated genes, including BRAF, RAS, TP53, PTEN, and TERT, whereas in the study by Angell et al., most of the samples were positive for BRAF, RAS, and TSHR mutations. Notably, even among thyroid samples that did not carry TERT, TP53, and other rare but clinically relevant mutations, 47/181 (26%) of mutations were missed when using RNA-Seq (9).

The second reason for missing some genetic variants in the RNA-Seq data is that not all genes are well expressed in the source tissue, and some loss-of-function mutations may lead to decreased stability and increased degradation of respective mRNA molecules. The low number of copies of mRNA
the tissue samples. This is because FNA samples frequently contain a significant proportion of nonneoplastic cells, including inflammatory and stromal cells, in addition to normal thyroid cells adjacent to the tumor nodule. In the tissue samples, most of these nonneoplastic cells can be excluded before molecular analysis by selecting the most cellular tumor areas using microscopic guidance. The higher fraction of normal cells in FNA samples would lead to lowering the mutant allelic frequency, which, as discussed earlier, decreases the chance for mutations to be detected by RNA-Seq.

Finally, the findings of this study suggest that allelic frequency of mutations cannot be reliably calculated from the RNA-Seq data. This is not surprising in light of the known variability in expression levels of the normal and mutant alleles. Knowing the allelic frequency of mutations may help to provide a more specific cancer probability assessment in the tested nodules (35), although such calculations may be inaccurate based on the data generated by RNA-Seq.

In summary, the results of this study, as well as previous reports, indicate that a significant proportion of coding mutations and all noncoding TERT promoter mutations are missed by sequencing of RNA isolated from thyroid samples, similar to other tissue types. This should be taken into account when RNA-Seq is used in clinical practice for diagnosis, prognosis, and selection of targeted therapies for thyroid and other cancer types.

Author Disclosure Statement

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3

References

1. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M, Schuff KG, Sherman SI, Sosa JA, Steward DL, Tuttle RM, Walterosky L 2016 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer. Thyroid 26:1–133.

2. Durante C, Granit G, Lamartina L, Filetti S, Mandel SJ, Cooper DS 2018 The diagnosis and management of thyroid nodules: a review. JAMA 319:914–924.

3. Xing M 2019 Genetic-guided risk assessment and management of thyroid cancer. Endocrinol Metab Clin North Am 48:109–124.

4. Chintakuntlaivar AW, Foote RL, Kasperbauer JL, Bible KC 2019 Diagnosis and management of anaplastic thyroid cancer. Endocrinol Metab Clin North Am 48:269–284.

5. O’Brien TD, Jia P, Xia J, Saxena U, Jin H, Vuong H, Kim P, Wang Q, Aryee MJ, Mino-Kenudson M, Engelmann JA, Le LP, Iafrate AJ, Heist RS, Pao W, Zhao Z 2015 Inconsistency and features of single nucleotide variants detected in whole exome sequencing versus transcriptome sequencing: a case study in lung cancer. Methods 83:118–127.

6. Ku CS, Wu M, Cooper DN, Naidoo N, Pawitan Y, Pang B, Jiacoppeta B, Soong R 2012 Exome versus transcriptome sequencing in identifying coding region variants. Expert Rev Mol Diagn 12:241–251.

7. Cirulli ET, Singh A, Shianna KV, Ge D, Smith JP, Maia JM, Heinenzen EL, Goedert JJ, Goldstein DB, Center for HIVAVI 2010 Screening the human exome: a comparison of whole genome and whole transcriptome sequencing. Genome Biol 11:R57.

8. Piskol R, Ramaswami G, Li JB 2013 Reliable identification of genomic variants from RNA-seq data. Am J Hum Genet 93:641–651.

9. Angell TE, Wirth LJ, Cabanillas ME, Shindo ML, Cibas ES, Babiarz JE, Hao Y, Kim SY, Walsh PS, Huang J, Kloos RT, Kennedy GC, Waguespack SG 2019 Analytical and clinical validation of expressed variants and fusions from the whole transcriptome of thyroid FNA samples. Front Endocrinol (Lausanne) 10:612.

10. Rennert H, Eng K, Zhang T, Tan A, Xiang J, Romanel A, Kim R, Tam W, Liu YC, Bhinder B, Cyerta J, Beltran H, Robinson B, Mosquera JM, Fernandes H, Demichelis F, Shoner A, Kluk M, Rubin MA, Elemento O 2016 Development and validation of a whole-exome DNA sequencing test for simultaneous detection of point mutations, indels and copy-number alterations for precision cancer care. NPJ Genom Med 1:e16019.

11. Li H, Durbin R 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760.

12. Nikiforova MN, Nikitski AV, Panebianco F, Kaya C, Yip L, Williams M, Chiosea SI, Seethala RR, Roy S, Condello V, Santana-Santos L, Wald AI, Carty SE, Ferris RL, El-Naggar AK, Nikiforov YE 2019 GLIS rearrangement is a genomic hallmark of hyalinizing trabecular tumor of the thyroid gland. Thyroid 29:161–173.

13. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA 2010 The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20:1297–1303.

14. Clarke L, Fairley S, Zheng-Bradley X, Streeter I, Perry E, Lowy E, Tasse AM, Flicek P 2017 The international genomic sample resource (IGSR): a worldwide collection of genome variation incorporating the 1000 Genomes Project data. Nucleic Acids Res 45:D854–D859.

15. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare D, Bell I, Bindal N, Boutselakis H, Cole CG, Creighton C, Dawson E, Fish P, Harsha B, Hathaway C, Jege SC, Kok CY, Noble K, Ponting L, Ramshaw CC, Rye CE, Speedy HE, Stefancsik R, Thompson SL, Wang S, Ward S, Campbell PJ, Forbes SA 2019 COSMIC: the catalogue of somatic mutations in cancer. Nucleic Acids Res 47:D941–D947.

16. Wang K, Li M, Hakonarson H 2010 ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 38:e164.
17. Beltran H, Eng K, Mosquera JM, Sigaras A, Romanel A, Rennert H, Kossai M, Pauli C, Faltas B, Fontugne J, Park K, Banfielder J, Prandi D, Madhukar N, Zhang T, Padilla J, Greco N, McNary TJ, Herrscher E, Wilkes D, MacDonald TY, Xue H, Vacic V, Emde AK, Oschwald D, Tan AY, Chen Z, Collins C, Gleave ME, Wang Y, Chakravarty D, Schiffman M, Kim R, Campagne F, Robinson BD, Nanus DM, Tagawa ST, Xiang JZ, Smogorzewska A, Demicheli F, Rickman DS, Shoner A, Elemento O, Rubin MA 2015 Whole-exome sequencing of metastatic cancer and biomarkers of treatment response. JAMA Oncol 1:466–474.

18. Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrtja J, Marotz C, Giannopoulou E, Chakravarti BV, Varambally S, Tomlins SA, Nanus DM, Tagawa ST, van Allen EM, Elemento O, Shoner A, Garraway LA, Rubin MA, Demicheli F. 2016 Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nat Med 22:298–305.

19. Chakravarty D, Shoner A, Nair SS, Giannopoulou E, Li R, Hennig S, Mosquera JM, Pauwels J, Park K, Kossai M, MacDonald TY, Fontugne J, Erho N, Vergara IA, Ghadessi M, Davicioni E, Jenkins RB, Palanisamy N, Chen Z, Nakagawa S, Hirose T, Bander NH, Beltran H, Fox AH, Elemento O, Rubin MA 2014 The oestrogen receptor alpha-regulated IncRNA NEAT1 is a critical modulator of prostate cancer. Nat Commun 5:5383.

20. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR 2013 STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21.

21. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S 2009 The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079.

22. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L 2012 Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7:562–578.

23. Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knigges-Langenburg G, Lassmann T, Carninci P, Brown JB, Livio PG, Gonzalez JM, Thomas M, Davis CA, Shiekhattar R, Gingeras TR, Hubbard TJ, Nodredefame C, Harrow J, Iafrate AJ, Lander E, Birney E, Hubbard TJ, Iafrate AJ, Lander E, Birney E, et al. 2012 The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res 22:1775–1789.

24. Johnson WE, Li C, Rabinovic A. 2007 Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8:118–127.

25. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD 2012 The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics 28:882–883.

26. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, Banks E, Garimella KV, Altschuler D, Gabriel S, DePristo MA 2013 From FastQ data to high confidence variant calls: the genome analysis toolkit best practices pipeline. Curr Protoc Bioinformatics 43:11.10.1–11.10.33.

27. Nikiforova MN, Mercurio S, Wald AI, Barbi de Moura M, Callenberg K, Santana-Santos L, Gooding WE, Yip L, Ferris RL, Nikiforov YE 2018 Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. Cancer 124:1682–1690.

28. Jennings LJ, Arcila ME, Corless C, Kamel-Reid S, Lubin JM, Pfeifer J, Temple-Smolkin RL, Voelkerding K, Nikiforova MN 2017 Guidelines for validation of next-generation sequencing-based oncology panels: a joint consensus recommendation of the association for molecular pathology and college of American pathologists. J Mol Diagn 19:341–365.

29. Gougeon DJ 2014 Regression: models, methods and applications. Curr Rev Acad Libr 51:1442.

30. Song YS, Lim JA, Choi H, Won JK, Moon JH, Cho SW, Lee KE, Park YJ, Yi KH, Park DJ, Seo JS 2016 Prognostic effects of TERT promoter mutations are enhanced by coexistence with BRAF or RAS mutations and strengthen the risk prediction by the ATA or TNM staging system in differentiated thyroid cancer patients. Cancer 122:1370–1379.

31. Ganly I, Makarov V, Deraje S, Dong Y, Reznik E, Seshan V, Nanjungad G, Eng S, Bose P, Kuo F, Morris LGT, Landa I, Carrillo Albornoz PB, Riaz N, Nikiforov YE, Patel K, Umbricht C, Zeiger M, Kebebew E, Sherman E, Ghossein R, Fagin JA, Chan TA 2018 Integrated genomic analysis of Hurthle cell cancer reveals oncogenic drivers, recurrent mitochondrial mutations, and unique chromosomal landscapes. Cancer Cell 34:256.e257–270.e255.

32. Gopal RK, Kubler K, Calvo SE, Polak P, Livitz D, Rosebrock D, Sadow PM, Campbell B, Donovan SE, Amin S, Gigliotti BJ, Grabarek Z, Hess JM, Stewart C, Braunein LZ, Arndt PF, Mordecai S, Shih AR, Chaves F, Zhan T, Lubitz C, Kim J, Istrate AJ, Wirth L, Parangi S, Leshchiner I, Daniels GH, Mootha VK, Dias-Santagata D, Getz G, McFadden DG 2018 Widespread chromosomal losses and mitochondrial DNA alterations as genetic drivers in Hurthle cell carcinoma. Cancer Cell 34:242.e245–255.e245.

33. Karanamurthy A, Panebianco F, S JH, Vorhauer J, Nikiforova MN, Chiosea S, Nikiforov YE 2016 Prevalence and phenotypic correlations of EIF1AX mutations in thyroid nodules. Endocr Relat Cancer 23:295–301.

34. Palacios R, Gazave E, Fagin JA, Chan TA 2018 Integrated genomic analysis of Hurthle cell cancer reveals oncogenic drivers, recurrent mitochondrial mutations, and unique chromosomal landscapes. Cancer Cell 34:242.e245–255.e245.

35. Steward DL, Carty SE, Sippel RS, Yang SP, Sosa JA, Sipos B, Bardelli A, Ferris RL, Nikiforov YE 2018 Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. Cancer 124:1682–1690.

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