# Molecular profile of Hürthle cell carcinomas: recurrent mutations in the Wnt/β-catenin pathway

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## Abstract

*Objective:* Genomic alterations in Hürthle cell carcinomas (HCC) include chromosomal losses, mitochondrial DNA mutations, and changes in the expression profile of the PI3K-AKT-mTOR and Wnt/β-catenin pathways. This study aimed at characterizing the mutational profile of HCC.

*Methods:* Next-generation sequencing (NGS) of 40 HCC using a 102-gene panel including, among others, the MAPK, PI3K-AKT-mTOR, Wnt/β-catenin, and Notch pathways. HCC was widely invasive in 57.5%, and lymph node and distant metastases were diagnosed in 5% and 7.5% of cases. During follow-up, 10% of patients presented with persistent/recurrent disease, but there were no cancer-related deaths.

*Results:* Genetic alterations were identified in 47.5% of HCC and comprised 190 single-nucleotide variants and 5 insertions/deletions. The Wnt/β-catenin pathway was most frequently affected (30%), followed by MAPK (27.5%) and PI3K-AKT-mTOR (25%). *FAT1* and *APC* were the most frequently mutated genes and present in 17.5%. *RAS* mutations were present in 12.5% but no *BRAF* mutation was found. There was no association between the mutational profile and clinicopathological features.

*Conclusions:* This series of HCC presents a wide range of mutations in the Wnt/β-catenin, MAPK and PI3K-AKT-mTOR pathways. The recurrent involvement of Wnt/β-catenin pathway, particularly mutations in *APC* and *FAT1*, are of particular interest. The data suggest that mutated *FAT1* may represent a potential novel driver in HCC tumorigenesis and that the Wnt/β-catenin pathway plays a critical role in this distinct thyroid malignancy.

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## Introduction

Hürthle cell carcinomas (HCC) represent about 3% of thyroid cancers. It is defined as a tumor composed of more than 75% of Hürthle cells - large cells with mitochondria-rich eosinophilic granular cytoplasm, large centrally located nuclei, and prominent nucleoli. It differs from Hürthle cell adenomas (HCA) by the presence of capsular and/or vascular invasion or metastasis (1). The latest edition of the WHO classification of tumors distinguished HCC from follicular thyroid cancer due to distinct clinical behavior and recent molecular findings (1). Hürthle cell carcinomas tend to be associated with poorer outcomes compared to papillary or follicular thyroid cancers (PTC,

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FTC). Retrospective analysis of a single-center experiences in the follow-up of HCC demonstrated 5-year cumulative probability of survival of 85%, which was worse in patients with distant metastases at diagnosis (24%) (2). In that study, clinical recurrence or death occurred in 44% of patients after initial response to therapy, but none of those had minimally invasive carcinomas (2). Moreover, despite improved survival rates attributed to radioactive iodine therapy (RAI) (3), the response of metastatic HCC to RAI is often very limited and not comparable to metastatic differentiated thyroid carcinomas (DTC) (2, 4, 5).

The molecular pathogenesis of HCC is less well characterized compared to PTC and FTC. Chromosomal changes and mitochondrial mutations are frequent events in the pathogenesis of HCC (6, 7). Transcriptome analysis revealed differences in the expression profiles between widely invasive HCC (HWIDE) and minimally invasive HCC (HMIN). The PI3K-AKT-mTOR and Wnt/β-catenin pathways appear to be highly active in HWIDE (8). Two recent landmark studies have provided significant insights into the genomic landscape of these neoplasias (9, 10). Widespread chromosomal losses, prevalent chromosomal 5 and 7 duplications, and recurrent mitochondrial DNA mutations were among the most prevalent findings (9, 10). A high rate of mitochondrial mutations affecting complex I subunits was observed in HCC. However, complex I alterations were also present in HCA (10), indicating that additional events are necessary for tumor progression. In these two seminal studies, the reported recurrent mutations in HCC were heterogeneous and different from each other (9, 10). Using the MutSig algorithm, the study by Ganly *et al.* reported 23 significantly mutated genes, including *EIF1AX* (mutated in 11% of HCC), *MADCAM1* (20%), *UBXN11* (9%) and *NRAS* (9%) (9), while the study by Gopal and colleagues identified frequent mutations in *DAXX* (17%), *TP53* (12%), *NRAS* (7%) and *NF1* (17%) (10). Of note, the characteristic mutations and rearrangements associated with PTC and FTC are rarely found in HCC (9, 10).

A more developed understanding of the somatic alterations in HCC remains of relevance and may facilitate the preoperative diagnosis, in particular, because commercially available molecular tests still fail in accurately distinguishing HCA from HCC despite improvements in recently updated versions (11, 12).

The aims of this study were to characterize the mutational profile in HCC using a next-generation sequencing (NGS) gene panel covering genes in pathways known to be active in HCC, or involved in other thyroid tumors, and to correlate the molecular findings with clinical-pathological features and outcomes.

## Subjects and methods

### Subjects

Formalin-fixed paraffin-embedded (FFPE) surgical specimens of HCC and HCA were selected for genomic analyses. The samples were obtained from patients who underwent thyroidectomy at the *Hospital das Clinicas* and the *Instituto do Cancer do Estado de Sao Paulo* between 1998 and 2017. Patient data, treatment modalities, and outcomes were collected through a retrospective chart review. The response to therapy was described according to the American Thyroid Association classification (13). The data were anonymized prior to further analysis. The study was approved by the Research Ethics Committee of the University of Sao Paulo.

An experienced pathologist reviewed all specimens to confirm the diagnosis and to select appropriate tissue samples for DNA extraction. Tumors were classified as: (1) HMIN, if it was encapsulated, harboring <4 foci of vascular invasion (within or immediately outside the tumor capsule), and without either gross invasion of the tumor capsule or vascular invasion of extrathyroid vessels and (2) HWIDE, if the tumor was encapsulated with ≥4 foci of vascular invasion, presence of widespread infiltration of adjacent thyroid or extrathyroidal tissue, and/or the presence of extrathyroidal vascular invasion.

### Next-generation sequencing

DNA was extracted from 40 HCC and their respective normal thyroid tissues, as well as 10 unpaired HCA, using a commercially available QIAamp® DNA FFPE Tissue kit (Qiagen, Düsseldorf, Germany). The nucleic acid concentration was determined with a Qubit® dsDNA High Sensitivity assay (Life Technologies, Carlsbad, USA), and DNA quality was determined with the DNA integrity score obtained from real-time PCR analysis.

Next-generation sequencing was performed using the SureSelect$^{XT}$HS Target Enrichment System protocol (Agilent Technologies, Santa Clara, USA). The customized capturing panel was developed to include 102 genes within pathways previously described to play important roles in thyroid tumorigenesis such as the MAPK, PI3K-AKT-mTOR, Wnt/β-catenin and Notch pathways (Table 1). NGS libraries were prepared using SureSelect$^{XT\ HS}$ library preparation kit according to the manufacturer's protocol. DNA was sheared using focused-ultrasonicator (Covaris), and the fragments were end-paired, adenylated, ligated to Illumina sequencing adapters, and amplified

**Table 1** Genes included in the next-generation sequencing panel for the analysis of Hürthle cell thyroid neoplasias.

| Pathways | Genes |
|---|---|
| MAPK | *ALK, BRAF, CCDC6, EGFR, EML4, ERBB2, ETV6, FGFR1, FGFR2, FGFR3, FLT3, FLT4, HRAS, KDR, KIT, KRAS, MAP2K1, MAP2K2, MAPK1, MAPK3, MET, NCOA4, NRAS, NTRK1, NTRK3, PAX8, PDGFRA, PDGFRB, PPARG, RET, ROS1* |
| PI3K-AKT-mTOR | *AGAP2, AKT1, AKT2, FOXO1, INPP5D, IRS1, IRS2, IRS4, MTOR, NFKB1, PDPK1, PIK3CA, PIK3CG, PIK3R1, PIK3R4, PIK3R5, PTEN, RHEB, RICTOR, SRC, STK11, TSC1, TSC2* |
| Wnt/β-catenin | *AMER1, APC, AXIN1, AXIN2, CCND1, CDK4, CSNK1A1, CSNK1D, CSNK1E, CTNNB1, CYLD, DKK1, DVL1, DVL2, FAT1, FLCN, FZD1, GNAS, GSK3B, HNF1A, JAK2, LRP5, LRP6, PPP2R1A, RNF43, SFRP1, TCF4, ZNRF3* |
| Notch | *FBXW7, MIR146A, MYC, NOTCH1, NOTCH2, NUMB* |
| Others | *BCR, DICER1, KEAP1, MAP4, MEN1, NFE2L2, NDUFA13 (GRIM19), SLC5A5, TERT* promoter, *TIMM44, TP53, TRIM62, TSHR, TWIST1* |

by PCR. Hybridization capture was performed using the SureSelect$^{XT\ HS}$ capture probe set, and captured libraries were enriched by PCR. Final libraries were quantified using the Agilent qPCR NGS Library Quantification kit, and the quality was analyzed by the Agilent 2200 TapeStation System. Normalized libraries were sequenced on the NextSeq (Illumina Inc, San Diego, EUA) using paired-end 2x 125-bp cycles. The average depth of coverage obtained was 1,111X (median 364.5, range 2.49–18 771.14). Four HCC samples with a mean coverage <50 times were maintained despite the low coverage because HCC is a rare malignancy and these cases had mutations identified by at least two somatic variant callers, including one *NRAS* mutation.

Raw-sequencing data (FASTQ files) were aligned to the hg19+decoy human genome build using *bwa* (14). Quality score recalibration and realignment around indels were performed using the Genome Analysis Toolkit (version 3.2.2, broadinstitute.org/gatk) (15). FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Qualimap (16) were used to assess the quality of the reads and sequencing coverage metrics. NGSCheckMate (17) was used to assess normal-tumor pairing.

Somatic alterations from normal-matched samples were independently identified by three callers: Lofreq (18), Mutect2 (19) and Lancet (20). Variants were included when recognized by at least two callers. In unpaired tumors, the single-sample somatic variant caller Pisces (https://github.com/Illumina/Pisces) was used to search for filtered and known mutations in the following genes: *FAT1, APC, KRAS, HRAS, NRAS, BRAF, TERT* promoter, *TP53, PTEN, MEN1,* and *TSHR*.

In this analysis, variants present in ≥5% of the reads and in <0.5% of individuals from the 1000 genomes database were included. All variants were manually reviewed using the Integrative Genomics Viewer (IGV) (21).

Genetic variants valid on IGV were grouped in the following categories: (i) known driver mutations involved in thyroid carcinogenesis; (ii) alterations previously described in the COSMIC70 database; (iii) frameshift or stop codon mutations; (iv) single-nucleotide variants (SNV) predicted as deleterious by one or more *in silico* tools (Polyphen, Mutation Taster, PROVEAN, and FATHMM); and (v) all the other genomic alterations.

The association between molecular alterations, pathological features (HWIDE or HMIN), and outcomes (lymph nodes or distant metastases, recurrent/persistent disease and response to therapy) were assessed.

### Statistical analysis

Data were processed using IBM SPSS Statistics for Windows version 24.0 (IBM, Armonk, NY). Two-tailed p values were used and p values < 0.05 were considered statistically significant. Categorical variables are presented as absolute and relative (percentages) frequencies. Differences were evaluated by Pearson's chi-square test and Fisher's exact test when appropriate. Continuous variables are presented as mean ± S.D. or median (range). Differences among studied subgroups were determined using Student's *t*-test if presenting normal distribution, and the Mann–Whitney *U* test for non-normal distributions.

## Results

### Clinical and pathological characteristics of HCC

A total of 23 patients with HWIDE and 17 with HMIN were selected for analysis. Clinical and pathological features are presented in Table 2. The mean HCC size was 5.2 ± 3.0 cm, and tumors were significantly larger among patients with HWIDE ($P = 0.014$). Lymph node metastases were diagnosed in 5% of subjects, and distant metastases in 7.5%. RAI therapy was performed in 78.4% of the patients. After a mean follow-up period of 69.6 ± 57.4 months, 10% of patients had persistent/recurrent disease.

**Table 2** Clinical features of patients with Hürthle cell carcinomas, HWIDE and HMIN.

| | HWIDE (*n* = 23) | HMIN (*n* = 17) | *P*-value |
|---|---|---|---|
| Age (years) | 58.6 ± 16.2 | 52.3 ± 12.5 | 0.212 |
| Female (%) | 17 (77.3) | 14 (82.4) | 0.697 |
| Tumor size (cm) | 6.1 ± 3.1 | 3.8 ± 2.1 | 0.014 |
| Radioiodine treatment (%)* | 19 (86.4) | 10 (66.7) | 0.153 |
| Lymph node metastasis (%)* | 2 (9.1) | – | 0.230 |
| Distant metastasis (%)* | 3 (13.6) | – | 0.136 |
| Recurrence and/or persistence (%)* | 4 (18.2) | – | 0.080 |
| Incomplete biochemical/structural response at final evaluation (%)* | 4 (18.2) | – | 0.091 |
| Follow-up (months) | 51.1 ± 43.7 | 96.9 ± 65.5 | 0.015 |

*Percentage refers to patients whose follow-up data were available.

Metastases, recurrences, as well as incomplete responses to therapy were restricted to patients with HWIDE. There was no cancer-related death.

## Somatic mutations in HCC

Somatic alterations were identified in 19 of the 40 (47.5%) HCC cases, 9 HWIDE and 10 HMIN. Altogether, we identified 190 somatic SNVs and 5 insertions/deletions (indels; Supplementary Table 1 (see section on Supplementary materials at the end of the article)). These SNVs included 181 nonsynonymous variants, 8 premature stop mutations, and 1 synonymous variant. In HWIDE, the median number of mutations per tumor was 11 (ranging 1–88), whereas HMIN had a median of 1.5 (1–5) mutations per tumor ($P = 0.105$).

Mutations in the pathways covered by the panel were mainly found among HWIDE cases (Table 3). The Wnt/β-catenin was the most frequently mutated pathway (Fig. 1), followed by the MAPK and the PI3K-AKT-mTOR pathways (Fig. 2).

*FAT1* and *APC* were the most frequently altered genes (Fig. 3). The *APC* gene harbored 18 mutations in 7 (17.5%) HCCs, 5 of which were HWIDE. Two *APC* mutations, p.R805Q and p.E1064*, have been previously described in colon carcinomas. The other mutations were predicted to be deleterious by *in silico* tools, in particular, the p.P2747L *APC* mutation which was detected in 2 HCCs and 1 HCA (Table 4).

There were 12 somatic *FAT1* mutations in 7 HCCs, 5 of which were HWIDEs. All the mutations were in either the cadherin or the cytoplasmic domains. The p.R1268Q *FAT1* mutation detected in three HCCs has been previously identified in glioblastoma multiforme (22). Nine mutations were predicted to be deleterious by *in silico* tools and 1 premature stop codon mutation (p.W948X) was detected (Table 4). Three somatic *FAT1* variants identified in 6 other HCC cases were observed as germline polymorphisms in the 1000 Genomes database (rs77834784, rs3796648 and rs2304867), suggesting that these might be passenger events. One HCA harbored a *FAT1* mutation (p.I1127V) that was only predicted to be deleterious by Mutation Taster but not by the other used tools (Table 4). Five HCCs (4 HWIDEs and 1 HMIN) had concomitant mutations in both the *FAT1* and *APC* genes (Fig. 3).

Other recurrently mutated genes within the Wnt/β-catenin pathway included *AXIN2* (10%), *LRP5* (10%), *DVL2* (7.5%), *CSNK1D* (5%), *DVL1* (5%), *GNAS* (5%), *LRP6* (5%), *ZNRF3* (5%) and *AMER1* (5%) (Figs 1 and 3).

A frequently mutated gene in the PI3K-AKT-mTOR pathway was *MTOR* (12.5%). *MTOR* somatic mutations

**Table 3** Somatic mutations of signaling pathways in Hürthle cell carcinomas, HWIDE and HMIN.

| Pathway/Genes | HWIDE | | HMIN | | Total | |
|---|---|---|---|---|---|---|
| | Cases (*n* = 23) | Mutations (*n* = 174) | Cases (*n* = 17) | Mutations (*n* = 21) | Cases (*n* = 40) | Mutations (*n* = 195) |
| Wnt/β-catenin | 7 (30.4%) | 64 (36.8%) | 5 (29.4%) | 9 (42.9%) | 12 (30.0%) | 73 (37.4%) |
| *FAT1* | 5 (21.7%) | 10 (5.7%) | 2 (11.8%) | 2 (9.5%) | 7 (17.5%) | 12 (6.2%) |
| *APC* | 5 (21.7%) | 15 (8.6%) | 2 (11.8%) | 3 (14.3%) | 7 (17.5%) | 18 (9.2%) |
| MAPK | 6 (26.0%) | 40 (23.0%) | 5 (29.4%) | 5 (23.8%) | 11 (27.5%) | 45 (23.1%) |
| PI3K-AKT-mTOR | 7 (30.4%) | 41 (23.6%) | 3 (17.6%) | 6 (28.6%) | 10 (25.0%) | 47 (24.1%) |
| Notch | 4 (17.4%) | 11 (6.3%) | – | – | 4 (10.0%) | 11 (5.6%) |
| Other genes | 4 (17.4%) | 18 (10.3%) | 1 (5.9%) | 1 (4.8%) | 5 (12.5%) | 19 (9.7%) |

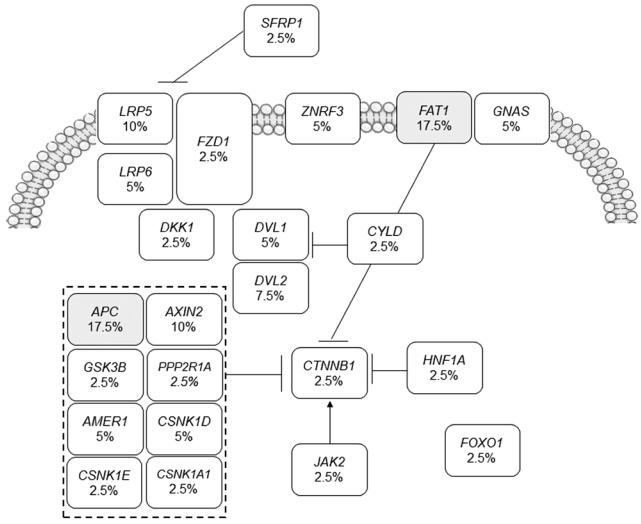


**Figure 1**
Frequency of Hürthle cell carcinomas harboring somatic mutations in genes from the Wnt/β-catenin pathway.

were observed in 12.5% of HCC, and these mutations affected the mTOR HEAT repeats region (p.R241H, p.E270K, p.A400V, and p.V1260I) and its kinase domain (p.G2337E) (Table 4). Other mutated genes within this pathway included *AGAP2* (15%), *AKT2* (10%), *PI3KR4* (10%), *IRS2* (7.5%) and *IRS1* (5%) (Figs 2 and 3).

The MAPK pathway harbored mutations in 27.5% of the HCCs included in this study, and the most frequently altered genes were *ALK* (10%), *FGFR3* (10%), *KDR* (10%), *KIT* (7.5%), *NRAS* (7.5%), *FLT4* (5%) and MET (5%) (Figs 2 and 3). *NRAS* was mutated in 3 HCCs; the p.Q61R *NRAS* mutation was identified in 2 carcinomas, and the p.Q61K *NRAS* mutation was identified in 1 carcinoma. The p.Q61R *NRAS* mutation was also observed in 2 HCAs. One HCC harbored a p.N54E *HRAS* mutation, and another carcinoma harbored the p.G12C *KRAS* mutation (Fig. 3).

Two novel mutations in the *TERT* promoter (chr5:1,295,594 G>A, chr5:1,295,494 A>G) were observed in two HWIDEs, both without lymph node or distant metastases. There were two non-metastatic HCC with mutations in the gene that encodes the sodium-iodide symporter, *SLC5A5/NIS*. One HMIN had a single mutation in *PTEN* (p.L281F) (Fig. 3). No mutations in *BRAF* or *TP53* were identified.

No significant association between the molecular profile and outcomes was observed. The 2 HCC associated with lymph node metastases did not harbor any mutations in the studied genes. Only a single tumor out of 3 with concomitant distant metastases (HWIDE8) harbored a mutation in *ROS1*, which is part of the MAPK pathway. This patient was the only subject with persistent disease and, hence, with an incomplete response to therapy, who had a mutation in the genes covered by our customized panel.

## Discussion

Despite growing knowledge about the genomic landscape of HCC, the driver events and the spectrum of genomic alterations responsible for this mitochondria-rich neoplasia that is distinct from other thyroid follicular cell-derived carcinomas remain incompletely characterized. The data presented here demonstrate recurrent mutations in the Wnt/β-catenin, MAPK and PI3K-AKT-mTOR pathways in HCC, involving, in particular, the *FAT1* and *APC* genes, which are implicated in Wnt/β-catenin activation.

Somatic mutations frequently associated with PTC and FTC are uncommon in HCC. According to the thyroid cancer datasets in The Cancer Genome Atlas (TCGA), *BRAF* mutations are present in up to 60% of PTCs (23, 24). Although Gopal *et al.* (10) identified 2 HCCs with *BRAF* mutations, the series presented here was negative for *BRAF* mutations, a finding that is in line with the data reported by Ganly *et al.* (9). *RAS* mutations

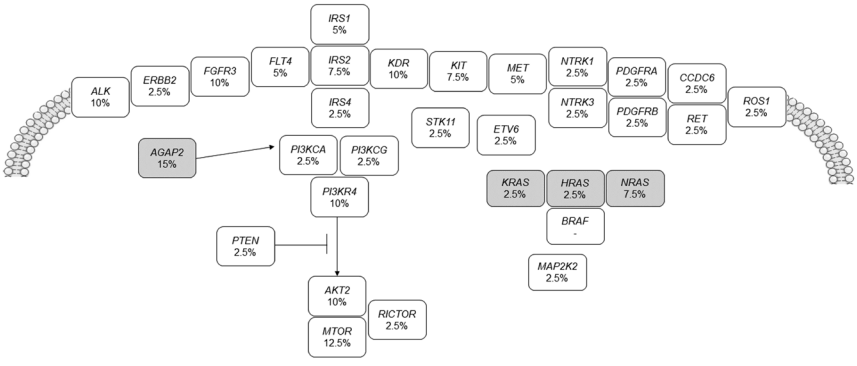


**Figure 2**
Frequency of Hürthle cell carcinomas harboring somatic mutations in genes from the MAPK and PI3K-AKT-mTOR pathways.

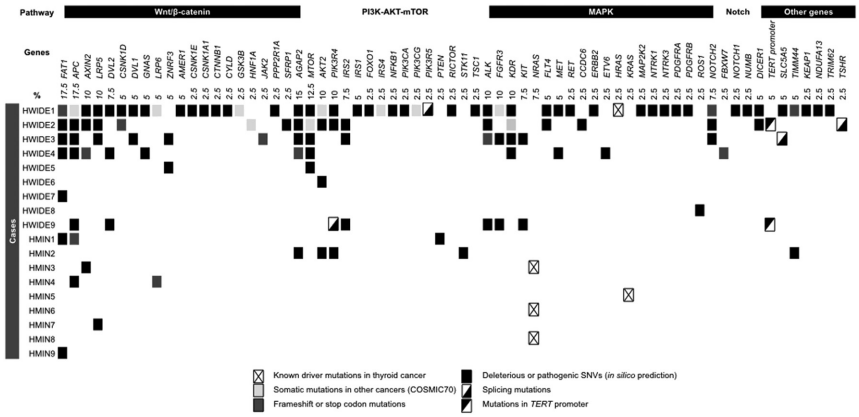


**Figure 3**
Genomic profile of Hürthle cell carcinomas according to the signaling pathways, pathogenicity and frequency of cases with mutations in each gene.

were observed in 5/40 HCC (12.5%) in our cohort, a rate that is similar to PTCs (24), but far less frequent than described in FTCs (48%) (8, 9, 25). However, the MAPK pathway is also relevant in HCC since a quarter of the carcinomas in our series harbored mutations in genes within this pathway.

The Wnt/β-catenin and PI3K-AKT-mTOR pathways were described as being highly active in HWIDE (8). This

**Table 4** Somatic *APC*, *FAT1* and *MTOR* mutations in Hürthle cell carcinomas.

| Gene/Exon | Point mutation | Amino-acid change | Pathogenicity | Allele frequency (%) |
|---|---|---|---|---|
| *APC* | | | | |
| 11 | c.C959T | p.S320L | Deleterious[a,b,c,d] | 12 |
| 11 | c.G1214A | p.R405Q | Deleterious[a,b,d] | 12 |
| 12 | c.G1375A | p.D459N | Deleterious[a,b,d] | 23 |
| 13 | c.G1426A | p.A476T | Deleterious[a,b,d] | 11 |
| 17 | c.C2302A | p.H768N | Deleterious[a,b,c] | 11 |
| 17 | c.G2392A | p.D798N | Deleterious[a,b,c,d] | 9–25 |
| 17 | c.G2414A | p.R805Q | COSM1432203[e] | 9 |
| 17 | c.G3190T | p.E1064* | COSM3428825[e] | 12 |
| 17 | c.G5062A | p.D1688N | Deleterious[b,c,d] | 44 |
| 17 | c.G5695A | p.E1899K | Deleterious[b,c] | 9 |
| 17 | c.C7028T | p.S2343F | Deleterious[a,b,c,d] | 10 |
| 17 | c.C7588T | p.R2530W | Deleterious[a,b,c,d] | 7–11 |
| 17 | c.G7628A | p.R2543K | Deleterious[b,c,d] | 12 |
| 17 | c.C8240T | p.P2747L | Deleterious[c] | 5–12 |
| *FAT1* | | | | |
| 2 | c.G2843A | p.W948* | Stop codon | 10 |
| 5 | c.G3832A | p.D1278N | Deleterious[a,b,c,d] | 11 |
| 5 | c.G3803A | p.R1268Q | COSM5946179[f] | 9–55 |
| 8 | c.G4420A | p.E1474K | Deleterious[a,b] | 20 |
| 10 | c.C4988T | p.A1663V | Deleterious[a,b,d] | 9 |
| 10 | c.C6698T | p.T2233I | Deleterious[a,b,d] | 8 |
| 10 | c.G7744A | p.V2582M | Deleterious[a,b,d] | 9 |
| 14 | c.C9557T | p.P3186L | Deleterious[a,b] | 14 |
| 19 | c.G10594A | p.D3532N | Deleterious[b] | 16 |
| 25 | c.G12880A | p.G4294R | Deleterious[b,c,d] | 11 |
| *MTOR* | | | | |
| 4 | c.504+5G>A | | Splicing | 11 |
| 5 | c.G641A | p.R214H | Deleterious[a,b,d] | 7 |
| 6 | c.G808A | p.E270K | COSM893952[g] | 11 |
| 8 | c.C1199T | p.A400V | Deleterious[a,b,d] | 18 |
| 25 | c.G3778A | p.V1260I | Deleterious[b,d] | 10 |
| 50 | c.G7010A | p.G2337E | Deleterious[a,b,c,d] | 13 |

The mutations were submitted to *in silico* analyses with various prediction tools, and queried for the presence in previous reports in other malignancies: (a) PROVEAN; (b) Mutation Taster; (c) FATHMM; (d) Polyphen; (e) Previously identified in colon carcinoma; (f) Previously identified in glioblastoma multiforme; (g) Previously identified in endometrial carcinoma.

is line with our findings with a total of 30% of all tumors with mutations in genes within the Wnt/β-catenin pathway, 58.3% of which were HWIDE. Moreover, 25% had mutations in PI3K-AKT-mTOR pathway genes, among which 70% were HWIDE. The *APC* gene was the most frequently altered gene, with 18 mutations in 17.5% of HCCs, although one HCA also had an *APC* mutation.

*APC* is a tumor suppressor gene known to activate the Wnt pathway when mutated, as thoroughly characterized in colorectal carcinomas (26). Apart from the p.P2747L *APC* mutation also identified in one of the studied adenomas, all mutations were predicted to be deleterious. Interestingly, *APC* mutations are associated with the cribriform-morular variant of PTC (CMV-PTC) in subjects with familial adenomatous polyposis (FAP) (27, 28). In the CMV-PTC, a second somatic hit is necessary in addition to the predisposing *APC* germline mutation for tumor development, an example of the Knudson two-hit concept. Whole-genome sequencing of CMV-PTC in FAP patients harboring germline mutations in *APC* revealed not only a somatic second hit mutation and/or LOH in *APC,* but also mutations in other genes such as *BRAF* p.V600E and *KMT2D* (28). It is possible that a monoallelic somatic variant in a gene different from *APC* may act as a driver in thyroid cells, or a trigger of malignant transformation of a cell that carries a pathogenic monoallelic *APC* variant (28). In our series, *APC* mutations consistently coexisted with other genetic alterations, in particular *FAT1* (5/7 *APC* mutated tumors), but also with mutations in genes from other pathways.

Our study suggests that *FAT1* might be a potential driver in HCC. *FAT1* was recurrently mutated, with 12 mutations in 17.5% of our HCCs. The PTC included in the TCGA (24) were negative for *FAT1* mutations, but Ganly *et al*. (9) also observed mutations in *FAT1* in a subset (7%) of HCCs, which is lower than the frequency observed in our cohort, a finding that further illustrates that the mutational profile of HCC is remarkably heterogeneous. *FAT1* mutations may also have a pathogenic role in medullary thyroid carcinomas (MTC). Genomic characterization of sporadic MTC revealed germline *FAT1* mutations in 4/18 tumors (22.2%), which also included copy number losses in chromosome 4, where *FAT1* is located (29).

FAT1 protein is involved in several cellular activities, such as cell adhesion, polarity and migration, in addition to its role in the Hippo and Wnt/β-catenin signaling pathways (22, 30). FAT1 negatively regulates β-catenin nuclear translocation through the cytoplasmic domain, restraining the Wnt/β-catenin pathway (22, 31, 32). Transcriptome analysis of tumors from TCGA network studies showed that low *FAT1* expression was associated with significant Wnt/β-catenin pathway enrichment in glioblastomas and ovarian cancers (22). Colorectal carcinomas harboring *FAT1* mutations had activated Wnt/β-catenin signaling despite the absence of mutations in other known drivers within this pathway (22). In breast and oral cancer, *FAT1* is downregulated (33, 34), while it is upregulated and associated with worse prognosis in acute lymphoblastic leukemia (35). Aberrant FAT1 processing occurs in melanoma (36) and mutations are present in pancreatic, head and neck squamous cell carcinomas (HNSCC), and, as illustrated in the present and previous studies, in HCC (9, 22, 30). The mechanisms through which *FAT1* mutations impair protein function appear to be diverse and require further clarification. *In vitro* studies in MTC cells demonstrated that FAT1 knockdown promotes cancer cell proliferation (29). The p.G4294R *FAT1* mutation identified in one of the HCCs in this study is located in the cytoplasmic domain, which is responsible for binding to β-catenin. Of note, in HNSCC, *FAT1* knockout did not change cell proliferation but increased migration and invasion (30). In in vitro study, FAT1-domain vectors (encoding amino acid residues 3438–4588) were transfected into FAT1-knockdown cells. The exogenous expression of FAT1-domains in transfected cells significantly decreased migration and invasion (30). Most of the *FAT1* mutations identified in this series are located in the extracellular (cadherin) domain. Functionally, the extracellular FAT1 domain is probably sufficient to reduce cell mobility; hence, mutations disrupting it are thought to favor cancer cell migration and invasion (30). Finally, Hürthle cells are characterized by the cytoplasmatic accumulation of abundant mitochondria (37). An in vitro study of smooth muscle cells lacking Fat1 (Fat1KO) demonstrated higher proliferation rates of these cells. In contrast, limiting mitochondrial respiration, either by pharmacologic or genetic interference targeting complex I, suppressed the growth advantage of Fat1KO cells, which suggest that a Fat1-mediated growth control mechanism is intrinsic to mitochondria (38). Therefore, although no functional evaluation was performed in the present study, based on these aforementioned studies, we hypothesize that inactivating FAT1 mutations in HCC might not only activate Wnt/β-catenin pathway and promote cell invasion, but also provide growth advantages to the mitochondria-rich Hürthle cells.

Regarding prognostic implications associated with *FAT1* mutations, none of the patients in the series presented here had unfavorable outcomes. In human

papillomavirus-negative HNSCC, glioma, and ovarian cancer, FAT1 mutations have been associated with better survival (22, 39, 40).

Although one of the studied HCAs harbored a FAT1 mutation, this finding does not discard *FAT1* as a potential driver since *RAS* and mitochondrial DNA mutations were also described in adenomas (9, 10). This finding suggests that *FAT1* mutations may represent an early event, and additional genetic alterations are postulated to be necessary to allow tumor progression.

In this study, *MTOR* was found to be a frequently mutated gene within the PI3K-AKT-mTOR pathway. mTOR regulates several cellular functions and is associated with cell proliferation and cancer progression (41). Mutations in *MTOR* were observed in 12.5% of this HCC cohort. The analyses of TCGA data only revealed mutation in *MTOR* in a single case among the 492 PTCs (0.2%) (24), while Murugan *et al.* showed that 1.2% (1/84) poorly differentiated thyroid carcinomas and 6.1% (2/33) anaplastic thyroid carcinomas (ATC) harbor *MTOR* mutations (42). Rare point mutations of *MTOR* have been reported in a few cancers, but only recently functional analyses demonstrated mTOR gain-of-function as a consequence of two novel mutations (42): H419R, located in the HEAT repeat domain, and G2359E, within the kinase domain, were identified in ATC and melanoma cell lines and both result in constitutive activation. The current model is that these mutations contribute to cancer aggressiveness by inducing invasion and metastasis (42). Of note, the HCC with *MTOR* mutations in our series were HWIDE tumors, although none of them was associated with distant metastases.

Finally, previous genomic studies focused on HCC identified mutations known to be associated with aggressive histology, such as *TERT* promoter or *TP53* mutations. They were not observed in the HCCs included here. The two novel mutations in the *TERT* promoter identified in our series are distinct from the C228T and C250T mutations and it remains currently unclear whether they have any impact on tumor development. In the comparison of primary to recurrent tumors, Gopal et al. observed independent mutations arising in distinct evolutionary branches, including *TERT* promoter and *TP53* mutations (10). Similarly, loss-of-function mutations in *SETD2*, a gene encoding a methyltransferase that trimethylates H3K36 and interacts with p53 to promote its stability, were observed in poorly differentiated metastases of two HCCs, also suggesting a role of SETD2 in HCC progression (43). As in other reports on the genomic characterization of HCC (9, 10), the present study is limited by sample size. However, both HWIDE and HMIN, as well as HCA, have been included. We restricted our analysis to primary tumors focusing on the identification of HCC driver mutations. Therefore, assertions about the acquisition of new mutations favoring metastatic dissemination were not possible.

In summary, the study presented here identified a mutational profile of HCC involving genes within the Wnt/β-catenin, MAPK and PI3K-AKT-mTOR pathways. The finding of recurrent involvement of the Wnt/β-catenin pathway, particularly mutations in *APC* and *FAT1*, are of particular interest. We assume that *FAT1* may represent a potential novel driver in HCC that may not only activate the Wnt/β-catenin pathway but also increase cell invasion and migration, and provide a growth advantage to mitochondria-rich Hürthle cells, a hypothesis that will need further corroboration through functional analyses in the future.

**Supplementary materials**

This is linked to the online version of the paper at https://doi.org/10.1530/EJE-20-0597.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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## References

1 *WHO Classification of Tumours of Endocrine Organs*. RV Lloyd, RY Osamura, G Klöppel, J Rosai (Editors) 4th Edition International Agency for Research on Cancer, Lyon, France, 2017.

2 Chindris AM, Casler JD, Bernet VJ, Rivera M, Thomas C, Kachergus JM, Necela BM, Hay ID, Westphal SA, Grant CS *et al.* Clinical and molecular features of Hurthle cell carcinoma of the thyroid. *Journal of Clinical Endocrinology and Metabolism* 2015 **100** 55–62. (https://doi.org/10.1210/jc.2014-1634)

3 Jillard CL, Youngwirth L, Scheri RP, Roman S & Sosa JA. Radioactive iodine treatment is associated with improved survival for patients with Hürthle cell carcinoma. *Thyroid* 2016 **26** 959–964. (https://doi.org/10.1089/thy.2016.0246)

4 Besic N, Schwarzbartl-Pevec A, Vidergar-Kralj B, Crnic T, Gazic B & Marolt Music M. Treatment and outcome of 32 patients with distant metastases of Hürthle cell thyroid carcinoma: a single-institution experience. *BMC Cancer* 2016 **16** 162. (https://doi.org/10.1186/s12885-016-2179-3)

5 Lopez-Penabad L, Chiu AC, Hoff AO, Schultz P, Gaztambide S, Ordonez NG & Sherman SI. Prognostic factors in patients with Hurthle cell neoplasms of the thyroid. *Cancer* 2003 **97** 1186–1194. (https://doi.org/10.1002/cncr.11176)

6 Corver WE, Ruano D, Weijers K, den Hartog WC, van Nieuwenhuizen MP, de Miranda N, van Eijk R, Middeldorp A, Jordanova ES, Oosting J *et al*. Genome haploidisation with chromosome 7 retention in oncocytic follicular thyroid carcinoma. *PLoS ONE* 2012 **7** e38287. (https://doi.org/10.1371/journal.pone.0038287)

7 Gasparre G, Porcelli AM, Bonora E, Pennisi LF, Toller M, Iommarini L, Ghelli A, Moretti M, Betts CM, Martinelli GN *et al*. Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic phenotype in thyroid tumors. *PNAS* 2007 **104** 9001–9006. (https://doi.org/10.1073/pnas.0703056104)

8 Ganly I, Ricarte Filho J, Eng S, Ghossein R, Morris LG, Liang Y, Socci N, Kannan K, Mo Q, Fagin JA *et al*. Genomic dissection of Hurthle cell carcinoma reveals a unique class of thyroid malignancy. *Journal of Clinical Endocrinology and Metabolism* 2013 **98** E962–E972. (https://doi.org/10.1210/jc.2012-3539)

9 Ganly I, Makarov V, Deraje S, Dong Y, Reznik E, Seshan V, Nanjangud G, Eng S, Bose P, Kuo F *et al*. Integrated genomic analysis of Hürthle cell cancer reveals oncogenic drivers, recurrent mitochondrial mutations, and unique chromosomal landscapes. *Cancer Cell* 2018 **34** 256.e5–270.e5. (https://doi.org/10.1016/j.ccell.2018.07.002)

10 Gopal RK, Kübler K, Calvo SE, Polak P, Livitz D, Rosebrock D, Sadow PM, Campbell B, Donovan SE, Amin S *et al*. Widespread chromosomal losses and mitochondrial DNA alterations as genetic drivers in Hürthle cell carcinoma. *Cancer Cell* 2018 **34** 242.e5–255.e5. (https://doi.org/10.1016/j.ccell.2018.06.013)

11 Nikiforova MN, Mercurio S, Wald AI, Barbi de Moura M, Callenberg K, Santana-Santos L, Gooding WE, Yip L, Ferris RL & Nikiforov YE. Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. *Cancer* 2018 **124** 1682–1690. (https://doi.org/10.1002/cncr.31245)

12 Patel KN, Angell TE, Babiarz J, Barth NM, Blevins T, Duh QY, Ghossein RA, Harrell RM, Huang J, Kennedy GC *et al*. Performance of a genomic sequencing classifier for the preoperative diagnosis of cytologically indeterminate thyroid nodules. *JAMA Surgery* 2018 **153** 817–824. (https://doi.org/10.1001/jamasurg.2018.1153)

13 Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M *et al*. 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* 2016 **26** 1–133. (https://doi.org/10.1089/thy.2015.0020)

14 Li H & Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009 **25** 1754–1760. (https://doi.org/10.1093/bioinformatics/btp324)

15 DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M *et al*. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics* 2011 **43** 491–498. (https://doi.org/10.1038/ng.806)

16 Okonechnikov K, Conesa A & García-Alcalde F. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics* 2016 **32** 292–294. (https://doi.org/10.1093/bioinformatics/btv566)

17 Lee S, Lee S, Ouellette S, Park WY, Lee EA & Park PJ. NGSCheckMate: software for validating sample identity in next-generation sequencing studies within and across data types. *Nucleic Acids Research* 2017 **45** e103. (https://doi.org/10.1093/nar/gkx193)

18 Wilm A, Aw PP, Bertrand D, Yeo GH, Ong SH, Wong CH, Khor CC, Petric R, Hibberd ML & Nagarajan N. LoFreq: a sequence-quality aware, ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets. *Nucleic Acids Research* 2012 **40** 11189–11201. (https://doi.org/10.1093/nar/gks918)

19 Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, Gabriel S, Meyerson M, Lander ES & Getz G. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nature Biotechnology* 2013 **31** 213–219. (https://doi.org/10.1038/nbt.2514)

20 Narzisi G, Corvelo A, Arora K, Bergmann EA, Shah M, Musunuri R, Emde AK, Robine N, Vacic V & Zody MC. Genome-wide somatic variant calling using localized colored de Bruijn graphs. *Communications Biology* 2018 **1** 20. (https://doi.org/10.1038/s42003-018-0023-9)

21 Thorvaldsdóttir H, Robinson JT & Mesirov JP. Integrative genomics viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in Bioinformatics* 2013 **14** 178–192. (https://doi.org/10.1093/bib/bbs017)

22 Morris LG, Kaufman AM, Gong Y, Ramaswami D, Walsh LA, Turcan Ş, Eng S, Kannan K, Zou Y, Peng L *et al*. Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation. *Nature Genetics* 2013 **45** 253–261. (https://doi.org/10.1038/ng.2538)

23 Danilovic DL, Lima EU, Domingues RB, Brandao LG, Hoff AO & Marui S. Pre-operative role of BRAF in the guidance of the surgical approach and prognosis of differentiated thyroid carcinoma. *European Journal of Endocrinology* 2014 **170** 619–625. (https://doi.org/10.1530/EJE-13-0944)

24 Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. *Cell* 2014 **159** 676–690. (https://doi.org/10.1016/j.cell.2014.09.050)

25 Nikiforova MN, Lynch RA, Biddinger PW, Alexander EK, Dorn 2nd GW, Tallini G, Kroll TG & Nikiforov YE. RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. *Journal of Clinical Endocrinology and Metabolism* 2003 **88** 2318–2326. (https://doi.org/10.1210/jc.2002-021907)

26 Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T & Nakamura Y. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Human Molecular Genetics* 1992 **1** 229–233. (https://doi.org/10.1093/hmg/1.4.229)

27 Cetta F, Montalto G, Gori M, Curia MC, Cama A & Olschwang S. Germline mutations of the APC gene in patients with familial adenomatous polyposis-associated thyroid carcinoma: results from a European cooperative study. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 286–292. (https://doi.org/10.1210/jcem.85.1.6254)

28 Nieminen TT, Walker CJ, Olkinuora A, Genutis LK, O'Malley M, Wakely PE, LaGuardia L, Koskenvuo L, Arola J, Lepisto AH *et al*. Thyroid carcinomas that occur in familial adenomatous polyposis patients recurrently harbor somatic variants in APC, BRAF, and KTM2D. *Thyroid* 2020 **30** 380–388. (https://doi.org/10.1089/thy.2019.0561)

29 Qu N, Shi X, Zhao JJ, Guan H, Zhang TT, Wen SS, Liao T, Hu JQ, Liu WY, Wang YL *et al*. Genomic and transcriptomic characterization of sporadic medullary thyroid carcinoma. *Thyroid* 2020 **30** 1025–1036. (https://doi.org/10.1089/thy.2019.0531)

30 Lin SC, Lin LH, Yu SY, Kao SY, Chang KW, Cheng HW & Liu CJ. FAT1 somatic mutations in head and neck carcinoma are associated with tumor progression and survival. *Carcinogenesis* 2018 **39** 1320–1330. (https://doi.org/10.1093/carcin/bgy107)

31 Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W & Holzman LB. Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. *EMBO Journal* 2004 **23** 3769–3779. (https://doi.org/10.1038/sj.emboj.7600380)

32 Tanoue T & Takeichi M. Mammalian Fat1 cadherin regulates actin dynamics and cell-cell contact. *Journal of Cell Biology* 2004 **165** 517–528. (https://doi.org/10.1083/jcb.200403006)

33 Lee S, Stewart S, Nagtegaal I, Luo J, Wu Y, Colditz G, Medina D & Allred DC. Differentially expressed genes regulating the progression of ductal carcinoma in situ to invasive breast cancer. *Cancer Research* 2012 **72** 4574–4586. (https://doi.org/10.1158/0008-5472.CAN-12-0636)
34 Nakaya K, Yamagata HD, Arita N, Nakashiro KI, Nose M, Miki T & Hamakawa H. Identification of homozygous deletions of tumor suppressor gene FAT in oral cancer using CGH-array. *Oncogene* 2007 **26** 5300–5308. (https://doi.org/10.1038/sj.onc.1210330)
35 de Bock CE, Ardjmand A, Molloy TJ, Bone SM, Johnstone D, Campbell DM, Shipman KL, Yeadon TM, Holst J, Spanevello MD *et al*. The Fat1 cadherin is overexpressed and an independent prognostic factor for survival in paired diagnosis-relapse samples of precursor B-cell acute lymphoblastic leukemia. *Leukemia* 2012 **26** 918–926. (https://doi.org/10.1038/leu.2011.319)
36 Sadeqzadeh E, de Bock CE, Zhang XD, Shipman KL, Scott NM, Song C, Yeadon T, Oliveira CS, Jin B, Hersey P *et al*. Dual processing of FAT1 cadherin protein by human melanoma cells generates distinct protein products. *Journal of Biological Chemistry* 2011 **286** 28181–28191. (https://doi.org/10.1074/jbc.M111.234419)
37 Máximo V, Lima J, Prazeres H, Soares P & Sobrinho-Simões M. The biology and the genetics of Hurthle cell tumors of the thyroid. *Endocrine-Related Cancer* 2012 **19** R131–R147. (https://doi.org/10.1530/ERC-11-0354)
38 Cao LL, Riascos-Bernal DF, Chinnasamy P, Dunaway CM, Hou R, Pujato MA, O'Rourke BP, Miskolci V, Guo L, Hodgson L *et al*. Control of mitochondrial function and cell growth by the atypical cadherin Fat1. *Nature* 2016 **539** 575–578. (https://doi.org/10.1038/nature20170)
39 Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature* 2011 **474** 609–615. (https://doi.org/10.1038/nature10166)
40 Kim KT, Kim BS & Kim JH. Association between FAT1 mutation and overall survival in patients with human papillomavirus-negative head and neck squamous cell carcinoma. *Head and Neck* 2016 **38** (Supplement 1) E2021–E2029. (https://doi.org/10.1002/hed.24372)
41 Laplante M & Sabatini DM. MTOR signaling in growth control and disease. *Cell* 2012 **149** 274–293. (https://doi.org/10.1016/j.cell.2012.03.017)
42 Murugan AK, Liu R & Xing M. Identification and characterization of two novel oncogenic mTOR mutations. *Oncogene* 2019 **38** 5211–5226. (https://doi.org/10.1038/s41388-019-0787-5)
43 Pecce V, Verrienti A, Abballe L, Carletti R, Grani G, Falcone R, Ramundo V, Durante C, Di Gioia C, Russo D *et al*. Loss of function SETD2 mutations in poorly differentiated metastases from two Hürthle cell carcinomas of the thyroid. *Cancers* 2020 **12**. (https://doi.org/10.3390/cancers12071892)

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