Somatic mutation profiling of hobnail variant of papillary thyroid carcinoma

Luca Morandi¹, Alberto Righi², Francesca Maletta³, Paola Rucci⁴, Fabio Pagni⁵,
Marco Gallo⁶, Sabrina Rossi⁷, Leonardo Caporali⁸, Anna Sapino⁹,
Ricardo V Lloyd¹⁰ and Sofia Asiola¹

¹Department of Biomedical and Neuromotor Sciences, Section of Anatomic Pathology 'M. Malpighi' at Bellaria Hospital, University of Bologna, Bologna, Italy

²Department of Pathology, Rizzoli Institute, (IRCCS), Bologna, Italy

³Department of Medical Sciences, University of Turin, Turin, Italy

⁴Section of Hygiene and Biostatistics, University of Bologna, Bologna, Italy

⁵Department of Pathology, University of Milano Bicocca, Monza, Italy

⁶Oncological Endocrinology Unit, Department of Medical Sciences, AOU Città della Salute e della Scienza di Torino, University of Turin, Turin, Italy

⁷Department of Pathology, Regional Hospital, Treviso, Italy

⁸Istituto delle Scienze Neurologiche di Bologna (IRCCS), Bellaria Hospital, Bologna, Italy

⁹Institute for Cancer Research and Treatment (IRCCS), Candiolo, Italy

¹⁰University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA

Correspondence
should be addressed
to S Asiola
Email
sofia.asiola3@unibo.it

Abstract

Hobnail variant of papillary thyroid carcinoma (HPTC) represents a recently described, aggressive and rare group of thyroid tumors with poorly understood pathogenesis. Molecular data about this group of cancers are few, and a more detailed molecular characterization of these tumors is needed. The main objective of the study is to define a comprehensive molecular typing of HPTC. Eighteen patients affected by HPTC, including eighteen primary tumors and four lymph node metastases, were screened for *NRAS*, *KRAS*, *HRAS*, *BRAF*, *TP53*, *PIK3CA*, *hTERT*, *PTEN*, *CDKN2A*, *EGFR*, *AKT1*, *CTNNB1* and *NOTCH1* gene mutations. Sequencing is conducted on the MiSEQ system, and molecular data are compared with clinical-pathologic data and follow-up. The patients include 14 women and 4 men. Ages range from 23 to 87 years. All 18 primary tumors of HPTC showed $\geq 30\%$ hobnail features. *BRAF* and *TP53* mutations are by far the most common genetic alterations in primary HPTC (72.2% and 55.6%, respectively), followed by *hTERT* (44.4%), *PIK3CA* (27.8%), *CTNNB1* (16.7%), *EGFR* (11.1%), *AKT1* (5.5%) and *NOTCH1* (5.5%). The mutational pattern in primary tumors and metastasis was usually maintained. Univariate Cox regression analyses with bootstrap procedure indicated a significantly increased mortality risk in patients harboring *BRAF* mutation and *BRAF* mutation associated with *TP53* and/or *PIK3CA* mutations. The detection of these multiple mutations appears to allow the identification of a subset of more aggressive tumors within the group and to bear information that should be useful for prognostic stratification of these patients including the planning of adjuvant therapy.

Key Words

- ▶ hobnail
- ▶ papillary thyroid carcinoma
- ▶ next-generation sequencing
- ▶ BRAF

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Introduction

Thyroid cancer is the most common type of endocrine malignancy. The vast majority of thyroid tumors originate from thyroid follicular cells and encompasses

well-differentiated papillary carcinoma and follicular carcinoma. Its prognosis is generally favorable, especially in well-differentiated thyroid carcinomas (DTCs) such

as papillary and follicular variants, which show survival rates of approximately 95% at 40 years (LiVolsi et al. 2004). Despite that, 15–20% of DTCs became radioiodine refractory (RAI-R) and until now no other therapeutic options have been effective (Haugen et al. 2016).

Recently, a new variant of papillary thyroid carcinoma (PTC), named ‘hobnail variant’ (HPTC) was described (Motosugi et al. 2009, Asioli et al. 2010, 2014, Bellevicine et al. 2012, Kakudo et al. 2012, Lino-Silva et al. 2012, Yang et al. 2013, Schwock et al. 2015, Ieni et al. 2016). It has a prevalence <2% and usually behaves more aggressively than classical PTC (Lino-Silva et al. 2012, Asioli et al. 2013, Amacher et al. 2015). The local recurrence rate is 23%, lymph node metastasis rate ranges from 60% to 75% and distant metastasis (including lung, brain and bone) rate ranges from 25% to 40% (Asioli et al. 2010, 2013, Chung et al. 2013). Overall survival rates are 69.1% and 64.1% at 5 and 10 years, respectively (Asioli et al. 2010, 2013, Chung et al. 2013). Disease-specific survival rates are 83, 71 and 54% at 5, 10 and 20 years after surgery, respectively (Lee et al. 2015).

Patients affected by HPTC seem to be good candidates for target therapies because they usually have locally advanced disease and distant metastases at the time of presentation and because the HPTC cells seem to be scarcely responsive to 131I treatment (Haugen et al. 2016).

To date, in PTC, the common known mutations are represented by several *RET/PTC* rearrangements (Romei & Elisei 2012), *BRAF* p.V600 mutation (Mathur et al. 2011, Romei & Elisei 2012) and more rarely gene amplifications and copy number gains involve genes encoding tyrosine kinase receptors (TKRs) (Abubaker et al. 2008, Liu et al. 2008, Santarpia et al. 2008) and epigenetic pathways including the aberrant methylation of thyroid-specific genes involved in iodine metabolism and/or of tumor suppressor genes, leading to the loss of radioiodine avidity and increased tumor growth, invasion and metastasization (Xing et al. 2015, Viola et al. 2016). Based on increase in our understanding of the molecular mechanisms underlying thyroid carcinogenesis, including that more aggressive thyroid cancers often show more than one gene alteration (Sobrinho-Simoes et al. 2008, Smallridge et al. 2009), innovative target therapies have been proposed in the last decade.

To date, molecular data in HPTCs are few, possibly reflecting the rarity of this entity. However, some previous reports have identified *BRAF* p.V600E mutation and a few have found *RET/PTC1* rearrangements consistent with PTC that has papillary architecture (Bellevicine et al. 2012, Yang et al. 2013, Asioli et al. 2014, Lubitz et al. 2014,

Ieni et al. 2016). A distinct molecular pathway of progression is poorly understood, and there is the need for a more detailed molecular characterization of these tumors. In this study, we report the results of our analysis of molecular alterations in a series of HPTCs that seem to improve risk stratification of these patients and to suggest the possible use of combined conventional and targeted therapies in this new entity.

Materials and methods

Ethics statement

All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by local ethics committee (Bologna-Imola CE-BI, number of study: 269/CE 2016 March 29th; Cod. CE: 16014). All information regarding the human material used in this study was managed using anonymous numerical codes.

Sample collection

Eighteen patients affected by HPTC, including eighteen primary tumors and four lymph node metastases, were selected from the databases of the Divisions of Pathology of the University of Turin (8 patients), University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin (2 patients), University of Milan (3 patients) and University of Bologna (5 patients retrieved from consult cases to SA) and included in the study.

The present series is constituted by previously unreported tumors with the exception of cases 2, 5 and 7, which have already been described in the series by Asioli and coworkers (cases 2 and 5 in Asioli et al. (2014) described as cases 1 and 2, respectively; case 7 in Asioli et al. (2013) described as case 10).

All cases have been classified according to the previous diagnostic criteria (>30% hobnail features in the papillary carcinoma component) (Asioli et al. 2010). Four cases of classic papillary thyroid tumors and a normal pooled genomic DNA (DNA Female pool, Cod. G1521 Promega) were included as internal control for mutation analysis.

Inclusion criteria were the availability of representative hematoxylin and eosin sections for histological review and paraffin blocks for immunohistochemistry and molecular testing. Data regarding age, sex, presentation, gross pathology and post-surgery radiotherapy (iodine radiotherapy) were obtained by review of clinical files and/or contacting the referring clinician. The pathologic

staging was assigned according to the Seventh Edition Cancer Staging Manual of the American Joint Committee on Cancer (Edge *et al.* 2010).

Disease persistence, recurrence and metastases were evaluated by medical record review, imaging studies or histopathologic examination of resected or recurrent tumors and/or metastases.

Immunohistochemistry

Sections were cut at 4 microns and stained with antibodies against thyroid transcription factor 1 (TTF-1) (8G7G3/1, 1:700; Dako), thyroglobulin (2H11/6E1 cocktail, 1/50BRD; Zymed, Waltham, MA, USA), p53 (DO7, 1:2000; Dako), mitochondria (JJ3-J, 1:100; Bio Genex, Fremont, CA, USA), Mucin 1 (MUC 1) (Ma695, 1:100; Novocastra, Milan, Italy), cytokeratin 7 (OB-TL12/30, 1:200; Dako), E-cadherin (4A2C7, 1:100; Zymed), β -catenin (E-5, 1:200; Santa Cruz) and Ki-67 (MIB-1, 1:300; Dako). Immunoreactions were performed according to the standard automated immunohistochemical procedure (Ventana XT autostainer).

Mutation analysis

Mutation analysis workflow is summarized in Fig. 1. DNA from formalin-fixed paraffin-embedded tissues was purified using the Simple Prep kit (4bases SA, Manno, Switzerland). Mutation analysis was performed by deep sequencing for the following genes previously described to be involved in thyroid carcinogenesis (Cancer Genome Atlas Research Network 2014, Charles *et al.* 2014, Lee *et al.* 2015): *KRAS* (Exons 2–4), *NRAS* (Exons 2–4), *HRAS* (Exons 2, 3), *BRAF* (Exon 15), *PIK3CA* (Exons 10, 21), *TP53* (Exons 4–9), *NOTCH1* (Exons 26, 27), *PTEN* (Exons 5–8), *CDKN2A* (Exons 1, 2), *EGFR* (Exons 18–21), *AKT1* (Exon 2), *CTNNB1* (Exon 3) and *hTERT* (Promoter). Locus-specific amplicon libraries with tagged primers were generated using target enrichment kits (4bases SA). Oligo primers included overhang adapters based on Nextera sequence at the 5', to be recognized by a second round of short PCR step to add Illumina (San Diego, CA, USA) P5/P7 sequencing adapters and sample-specific indices to samples. Six multiplex PCR tubes allowed parallel amplifications of all the above cited exons and *hTERT* promoter using *Phusion II* (Life Technologies), as a proofreading enzyme. Amplification products for each sample were mixed together and purified by AMPure XT (Agencourt-Beckman Coulter), quantified with the FluorometerQuantus (Promega) and then employed as template (100ng) for a second round

Collection of cases and selection of tumor area

DNA extraction

PCR enrichment of target regions (13 genes, 33 amplicons)

Library preparation: sample barcoding and pooling

Deep sequencing on MiSEQ (Illumina)

Data analysis using different software suites: BWA + VariantStudio, SEQPilot, NextGene

Figure 1

Mutational analysis work flow. Deep sequencing analysis involves the following steps.

of PCR (6 cycles) for barcoding (4bases SA). Amplicon products were purified with AgencourtAMPure XP beads, quantified with the FluorometerQuantus, pooled and loaded on MiSEQ (Illumina). Processed and quality-filtered reads from FASTQ files were analyzed with VariantStudio (Illumina), SEQPilot (JSI) and NextGene (SoftGenetics, State College, PA, USA). Only reads exceeding the Phred quality score of Q30 were retained for further bioinformatic analysis. Controls: DNA samples from normal pooled genomic DNA (Female pool, Cod. G1521 Promega), and cell lines known to carry a specific mutation were used as positive controls. Allele-specific locked nucleic acid quantitative PCR (ASLNAqPCR), as described previously (Morandi *et al.* 2012), was used as an orthogonal method to confirm the sensitivity and specificity of NGS results for *BRAF* (p.V600E) and *KRAS* (p.G12A, p.G12C, p.G12D, p.G12R, p.G12S, p.G12V and p.G13D) genes. Although the analytical sensitivity was detected to be less than 1%, as confirmed by ASLNAqPCR, we decided to set the clinical sensitivity to 3% as previously described (Nikiforov *et al.* 2014); thus, only mutations detected to cross this threshold were reported.

RET/PTC rearrangement

The *BRAF* mutation-negative patients (5 patients) were further tested for *RET/PTC* rearrangement. RNA was

extracted from the same paraffin blocks using RecoverAll kit (Life Technologies) as reported earlier (Asioli *et al.* 2010). In brief, RT-PCR for *RET/PTC1* and *RET/PTC3* was carried out using thyroid carcinoma with known *RET/PTC1* and *RET/PTC3* rearrangements as positive controls and omitting reverse transcriptase as negative controls. Primers used were as follows: *RET/PTC1*, 5'-ATT GTC ATC TCG CCG TTC-3' and *RET/PTC3*, 5'-TGG AGA AGA GAG GCT GTA TC-3'. Reverse primers were 5'-TGC TTC AGG ACG TTG AAC-3' for *RET/PTC1* and 5'-CGT TGC CTT GAC TTT TC-3' for *RET/PTC3*. The housekeeping gene *PGK* was used as an internal control.

Statistical analysis

Kaplan–Meier estimates of overall survival and disease-free survival were obtained. Univariate Cox regression analyses were used to compare the overall and disease-free survival between patients with and without a single mutation and between patients with and without multiple mutations. For multiple mutation analyses, we combined individuals with at least one *BRAF*, *TP53* or *PIK3CA* mutation and compared them with the rest of the sample. A bootstrap procedure was used to obtain robust estimates of the standard errors for Cox regression analyses. Statistical analysis was performed using the Prism 4 (GraphPad) and IBM SPSS, version 20.0. The significance level was set at $P < 0.05$.

Results

Clinicopathological data and immunohistochemical profile

The study cohort included 18 patients with HPTC. Clinical and histopathological features of the whole population are summarized in Table 1.

All patients underwent total thyroidectomy and cervical lymph nodes dissection, followed by radioiodine therapy (^{131}I between 100 and 200 mCi).

Six out of 18 patients had a good response to radioactive iodine with good uptake and shrinkage of metastatic diseases, when present. A poor response (evidence of recurrence) after an average of 50 months (see below) was evidenced in five patients who had RAI-responded to initial treatment with thyroglobulin levels $<1\text{ ng/mL}$. The remaining 7 patients evidenced a poor response to RAI without uptake. The patients' thyroglobulin levels were available in all but one patient and ranged between 0.3 ng/mL

Table 1 Clinical and histopathological characteristics of 18 hobnail papillary thyroid cancers.

	Number of cases	Total (%)
Gender		
Female	14	77.8
Male	4	22.2
Age (years)		
<45	3	16.7
≥45	15	83.3
Tumor size (cm)		
≤4	12	66.7
>4	6	33.3
Multifocality		
Yes	6	33.3
No	12	66.7
Extra-thyroidal extension		
Yes	16	88.9
No	2	11.1
Angiolymphatic invasion		
Yes	15	83.3
No	3	16.7
Mitosis per 10 high-powered fields (400×)		
<3	2	11.1
≥3	16	88.9
pT		
1	1	5.6
2	1	5.6
3	14	77.7
4	2	11.1
pN		
N0	5	27.8
N1a	2	11.1
N1b	11	61.1

and 102 ng/mL with a mean of 7.3 ng/mL ; median thyroglobulin levels showed a trend toward changing according to the radiological response (reduction, stabilization or progression).

Follow-up data were available for all patients with a mean of 40 months (range: 6–12 months). Five patients had recurrent disease after a mean of 50 months (range: 6–128 months) from the thyroidectomy. Eight patients developed metastatic disease, most commonly to the bone (5 cases), lung (2 cases) or mediastinum (1 case) after a mean of 68 months (range: 14–128 months) from the thyroidectomy. Recurrence and metastases to lymph nodes or distant organs showed a predominant hobnail pattern of growth similar to the primary tumor (Figs 2 and 3).

Four patients died of disease after a mean of 22 months. Seven patients remained alive with extensive disease after a mean follow-up of 49 months. Seven patients remained alive without disease after 42 months of follow-up.

The neoplastic cells showed variable thyroglobulin, TTF-1 and cytokeratin 7 immunoreactivity (Fig. 3). Ten out

Figure 2

Metastasis to distant organs including lung (A) and bone (B) and to lymph node (C), maintained predominant hobnail pattern of growth similar to the primary tumor. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0546>.

of 18 cases showed more than 25% of the neoplastic cell nuclei positive for p53 (Fig. 3) in the primary thyroid tumor and in lymph nodes metastases. E-cadherin and β -catenin are present in the basolateral or lateral site of neoplastic cells, and in three cases, β -catenin also showed nuclear co-expression in few cells (<5%). MUC1 staining showed the characteristic 'inside-out pattern' highlighted by hobnail features of the tumor cells. Only one case (case 12) showed focal positivity (20%) for the anti-mitochondria antibody. The mean of proliferative index Ki67 was 12%.

Molecular profile

DNA from all samples was successfully amplified in multiplex PCR, and an adequate library for NGS was obtained in all 18 patients. The mean read length was 183 base pairs and a mean coverage depth of 1203 was achieved, with 99.5% target bases covered more than 100 $\times$. No mutations were detected for normal pooled genomic DNA (Promega).

Pathogenic mutations in the 13 genes were found in 15 of 18 (83.3%) patients (Table 2). Of the

Figure 3

A HPTC case harboring BRAF, TP53 and PIK3A mutations shows papillary and micropapillary patterns of growth (A) with focal positivity for thyroglobulin (B), nuclear staining for TTF1 (C) and nuclear overexpression of p53 protein (D). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0546>.

Table 2 Summary of pathogenic mutations detected in a set of 13 genes and follow-up data.

Case	KRAS	HRAS	NRAS	BRAF	CDKN2A	TP53	PIK3CA	CTNNB1	hTERT	PTEN	EGFR	NOTCH1	AKT1	FU
1 T	WT	WT	WT	p.V600E (42%)	WT	p.S261N (21%)	p.H1060Y (4%)	p.E53K (28%)	WT	WT	p.A859T (43%)	p.D1681 (37%)	p.D32N (30%)	AWD (60)
1 N	WT	WT	WT	p.V600E (45%)	WT	p.S261N (20%)	p.H1060Y (4%)	p.E53K (20%)	WT	WT	p.A859T (39%)	WT	WT	
2 T	WT	WT	WT	p.V600E (35%)	WT	p.R282W (23%)	WT	p.G24E (15%)	WT	WT	WT	WT	WT	DOD (34)
2 N	WT	WT	WT	p.V600E (45%)	WT	p.R282W (17%)	WT	p.G24E (12%)	WT	WT	WT	WT	WT	
3 T	WT	WT	WT	WT	WT	p.A276T (6%)	WT	WT	123T>C (18%)	WT	WT	WT	WT	NED (39)
3 N	WT	WT	WT	p.A598V (15%)	WT	WT	WT	WT	102C>T (14%)	WT	WT	WT	p.G16E (18%)	
4 T	WT	WT	WT	p.V600E (15%)	WT	p.E287K (10%)	WT	p.D58N (21%)	WT	WT	WT	WT	WT	NED (36)
4 N	WT	WT	WT	p.V600E (11%)	WT	p.E287K (9%)	WT	p.D58N (13%)	WT	WT	p.R705K (11%)	p.V1577M (31%)	WT	
5	WT	WT	WT	p.V600E (24%)	WT	WT	WT	WT	124C>T (52%)	WT	WT	WT	WT	AWD (34)
6	WT	WT	WT	p.V600E (22%)	WT	p.R248Q (8%)	WT	WT	124C>T (19%)	WT	WT	WT	WT	DOD (7)
7	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NED (38)
8	WT	WT	WT	p.V600E (7%)	WT	p.Q192 STOP (10%)	p.E545K (15%)	WT	146C>T (36%)	WT	WT	WT	WT	DOD (12)
9	WT	WT	WT	p.V600E (19%)	WT	WT	p.E545K (14%)	WT	146C>T (38%)	WT	WT	WT	WT	AWD (128)
10	WT	WT	WT	p.V600E (25%)	WT	p.V274F (10%)	WT	WT	124C>T (34%)	WT	WT	WT	WT	AWD (74)
11	WT	WT	WT	p.V600E (30%)	WT	p.R248Q (11%)	p.E545K (22%)	WT	146C>T (15%)	WT	WT	WT	WT	AWD (14)
12	WT	WT	WT	p.V600E (20%)	WT	WT	WT	WT	WT	WT	WT	WT	WT	NED (110)
13	WT	WT	WT	WT	WT	p.V274F (16%)	WT	WT	WT	WT	p.L858R (36%)	WT	WT	AWD (6)
14	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NED (22)
15	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NED (8)
16	WT	WT	WT	WT	WT	p.R209I (4%)	WT	WT	WT	WT	WT	WT	WT	NED (41)
17	WT	WT	WT	p.V600E (25%)	WT	WT	p.E545K (23%)	WT	WT	WT	WT	WT	WT	DOD (36)
18	WT	WT	WT	p.V600E (14%)	WT	WT	WT	WT	124C>T (18%)	WT	WT	WT	WT	AWD (30)

Alternate variant frequency is indicated between brackets.
WT, wild type.

fifty-eight mutations found in these 22 cases of HPTC, including eighteen primary tumors and four lymph node metastases, forty-eight were missense, one was a premature stop codon and the remaining ones are single-base substitutions at the promoter of *hTERT*. Forty-six out of 49 genetic alterations found in coding regions are recorded in the COSMIC databases. The remaining three were not previously described in solid tumors and they were detected in *AKT1* (p.D32N, p.G16E) and *NOTCH1* (p.D1681N) genes. *BRAF* and *TP53* mutations were by far the most common genetic alterations in HPTC patients (72.7% and 55.6%, respectively). *BRAF* p.V600E mutation was the most common alteration found in our series. All the 15 samples positive for p.V600E by NGS were confirmed by ASLNAqPCR. Additionally, we identified a rarer variant, the p.A598V (case 3), which has been recorded in COSMIC database, and it was found pathogenic in thyroid cancer. Only five of 18 primary HPTC cases included in this study did not harbor any pathogenic mutations in *BRAF* gene.

TP53 mutations were detected in ten out of 18 primary HPTC cases, all of which have been included in the COSMIC and *TP53* database (IARC) and were considered pathogenic. Seven primary HPTC cases evidenced the simultaneous presence of both *BRAF* and *TP53* mutations.

PIK3CA mutations were found in five primary HPTC cases. *hTERT* was investigated from position -191 to -64 upstream from the ATG start site. In our series, eight cases out of 18 primary HPTC cases (44.4%) showed mutations mostly in the two damaging hot spots, at positions -124 and -146. *CTNNB1* was found to be mutated in three primary HPTC cases (16.7%), *EGFR* in two (11.1%) and *AKT1* and *NOTCH1* in one case (5.5%). Mutations of *CKDN2A* and *PTEN* were not detected. The mutational pattern in primary tumors and metastasis was for the most part maintained for cases 1 and 2, except for *NOTCH1* and *AKT1* in case 1, indicating clonality. The mutation frequency of *BRAF* was very close to 50% for cases 1 and 2 both for primary and metastasis. Cases 4–6, 8–12 and 17–18 showed a mutation frequency within a range between 7% and 30%. Among various gene mutations detected, those of *BRAF* presented the highest frequency supporting the fundamental role of this gene in HPTC variant and the presence of these mutations in the bulk of the tumor.

In case 3, only metastasis was involved in the rare *BRAF* p.A598V and *AKT1* p.G16E with 15% and 18% mutation allele frequency, respectively. Additionally *TP53* p.A276P (7% frequency) was detected only in

Figure 4

Kaplan–Meier estimates of overall survival in 10 HPTC with *BRAF*, *TP53* and/or *PIK3A* mutation vs the rest of sample ($N=8$).

primary lesion, and two different mutations were identified for *TERT*, proving tumor heterogeneity in this case (Table 2).

One patient showed mutations only for *BRAF* gene (p.V600E) in primary HPTC (case 12).

In the five patients without *BRAF* mutation, the *RET/PTC* rearrangement analyses were negative for *RET/PTC1* and *RET/PTC3* rearrangements in three patients. The remaining two patients did not have amplifiable cDNA.

Figure 4 shows the Kaplan–Meier estimates of overall survival in 10 HPTC with *BRAF*, *TP53* and/or *PIK3A* mutation vs the rest of sample ($N=8$).

Table 3 summarizes the results of univariate Cox regression analyses with bootstrap procedure that indicate a significantly increased mortality risk in patients harboring *BRAF* mutation and *BRAF* mutation associated with *TP53* and/or *PIK3CA* mutations. Specifically, in patients harboring *BRAF* mutation, the mortality risk was 5.4-fold compared with those not harboring the mutation, and it was 6.6-fold in those harboring the combination of mutations. None of the gene alterations considered, including *BRAF*, *TP53*, *PIK3CA*, *hTERT* and *CTNNB1*, was associated with a shorter disease-free survival.

Table 3 Hazard ratios (HR) and 95% confidence intervals (CI) estimated using a bootstrap procedure.

Genes	HR	95% CI		P
<i>BRAF</i>	5.394	4.204	6.917	0.001
<i>TP53</i>	1.596	0.190	13.404	0.233
<i>PIK3CA</i>	1.500	0.154	14.622	0.218
<i>CTNNB1</i>	1.095	0.112	10.745	0.907
<i>hTERT</i>	1.207	0.147	9.924	0.661
<i>BRAF</i> and (<i>TP53</i> and/or <i>PK3CA</i>)	6.605	4.217	10.348	0.001

Discussion

As the original description of HPTC as a separate entity among malignant thyroid tumors, several tumor series have been reported (Bellevicine et al. 2012, Asioli et al. 2013, 2014, Amacher et al. 2015, Lee et al. 2015). In this study, we performed immunohistochemical and molecular analyses of a large series of HPTCs selected using the strict previously described criteria (Asioli et al. 2013). The immunohistochemical profile of our series is consistent with data previously reported (Asioli et al. 2010). The peculiar and distinctive immunophenotype shown by HPTC, which consists of TTF-1 and thyroglobulin expressions (markers expressed in well-differentiated thyroid cancer and lost in ATC) and in the simultaneous overexpression of p53 protein (marker overexpressed in ATC), was confirmed from the molecular analysis for the gene *TP53*.

TP53 pathogenetic mutations were detected in 10 out of 18 primary HPTC cases, all of which have been included in the COSMIC and *TP53* database (IARC) and were considered pathogenic. All ten cases showed more than 25% of the neoplastic cell nuclei positive for p53 in the primary thyroid tumor, and we did not observe any peculiar p53 immunohistochemistry correlation with mutation status in either distribution and/or intensity. Nikiforova and coworkers (Nikiforova et al. 2013), using the sensitive NGS technique, reported β -catenin follicular thyroid carcinoma oncocyctic variant, and in 29.6% of ATC. They reported an increasing frequency of *TP53* mutations in dedifferentiating thyroid carcinomas. The same study reported the presence of mutations for *PIK3CA* in 11.1% of PTC and ATC and one case of ATC and one case of PTC showed concurrent mutation in *PIK3CA* and *TP53* (Nikiforova et al. 2013).

In addition, neoplastic cells of HPTC showed expression of E-cadherin and β -catenin in the basolateral or lateral site, and in three cases, we found mutations of *CTNNB1* and β -catenin nuclear co-expression in few neoplastic cells (<5%). Molecular data in our series found *CTNNB1* gene mutated in three patients (16.7%), both in primary and in metastatic tumor, confirming our previous data (Asioli et al. 2010). Considering *CTNNB1* gene mutations, Nikiforova and coworkers (Nikiforova et al. 2013) found only one positive case of ATC. Basolateral or lateral or nuclear expression of β -catenin is considered one of molecular event involved in epithelial-mesenchymal transition (EMT). EMT is an established pathologic mechanism in epithelial tumor progression, local invasion and metastasis that has been

implicated in HPTC (Masago et al. 2009). The elucidation of molecular mechanisms that govern EMT will offer new approaches and targets to restrain metastasis.

Our molecular results identify multiple alterations including an initial driver (*BRAF*) plus second hits (*TP53*, *TERT* and *PIK3CA*) that is not novel or unique to HPTC (Cancer Genome Atlas Research Network 2014), but this molecular subtype could be useful to better identify HPTCs that reflect its underlying signaling and differentiation properties, which has the potential to improve its pathological classification. Indeed, *BRAF* mutations were detected mostly in the bulk of tumor with a higher variant allele frequency (VAF) than those found in other genes such as *PIK3CA*, *TP53* or *CTNNB1* (Table 2). This mutational pattern revealed the fundamental role of *BRAF* as a driver, followed by *TP53*, and in a more restricted number of cases by *PIK3CA*, *CTNNB1* and *hTERT*.

For instance, the primary tumor of case 1 showed a VAF of 42% for *BRAF* p.V600E, 43% for *EGFR* p.A859T and 37% for *NOTCH1* p.D1681. This means that almost all the bulk of this tumor shared these mutations that can be considered early events in its evolution. Other mutations detected in *TP53*, *CTNNB1*, *AKT* and *PIK3CA* displayed lower VAF (Table 2), a typical sign of later events that involved a more limited number of tumor cells. In 3 out of 4 cases where metastasis was available (cases 1, 2 and 4), the same mutation pattern with a similar VAF was present in both primary tumor and metastasis, revealing that presumably these mutations were maintained over the time course of the tumor evolution, except for *NOTCH1* and *AKT1* mutations. Although our series is made of a limited number of cases, it may reveal that in these patients a single clone gave rise to lymph node metastasis maintaining driver mutations in *BRAF*, *TP53* or *PIK3CA*, and mutations in these genes are related to a more aggressive behavior. Activating mutations of *BRAF* and *PIK3CA* in cooperation has been described recently in anaplastic carcinoma (Charles et al. 2014), and treating these tumors with selective or multi-tyrosine kinase inhibitor such as sorafenib, may improve clinical outcome (Brose et al. 2014). Only case 3 exhibited a completely different mutational pattern between primary tumor and metastasis with 4 unmatched mutations. This means that the primary tumor is unrelated to lymph node metastasis, which may be derived from an occult undetected primary tumor or by a micro-clone not present in cells collected from the selected tumor tissue. The combined mutations in *BRAF*, *TP53* and/or *PIK3CA* genes by molecular profiling seem to be clinically relevant for the prognostic stratification

and treatment of these patients. *BRAF* p.V600E and the rarer variant p.A598V were found in most cases of our series. Accordingly, *BRAF* p.V600E mutation is the most prevalent point mutation in PTC being present in 36–69% of cases (Trovisco et al. 2005), and it was detected almost exclusively in papillary or mixed follicular/papillary thyroid carcinomas (Trovisco et al. 2005). Currently, poor prognostic indicators, such as extra-thyroid extension (Nikiforova et al. 2003), metastasis, higher tumor staging, tumor size and recurrence (Xing et al. 2015), were found associated with *BRAF* mutations as well as its association with a decrease in expression of several ‘thyroid-specific genes’ or ‘iodine-handling genes’. Previous studies reported that low radioactive iodine avidity are associated to *BRAF* mutations *in vitro* and *in vivo* (Chakravarty et al. 2011), and this should be the case for HPTC.

In the present series, *RET/PTC1* and *RET/PTC3* rearrangements were not detected in *BRAF* V600E-negative tumors, confirming our previous data (Asioli et al. 2010). Recently, Lubitz and coworkers (Lubitz et al. 2014), using SNaPshot analysis reported that two *BRAF* p.V600E-negative tumors out of twelve HPTC cases carried the *RET/PTC1* gene rearrangement as well as Ieni and coworkers (Ieni et al. 2016), using RT-PCR analysis, among the 4 *BRAF* p.V600E-negative cases out of eight HPTC cases reported in their study, only one exhibited *RET/PTC1* gene rearrangement.

hTERT mutations were found in 8 out of 18 patients with HPTC (44.4%) in this study, and similar data for poorly differentiated carcinomas (29%) and anaplastic carcinomas (33.3%) were described by other groups with a significant association with disease-specific mortality (Melo et al. 2014). The mutations in the current study occurred mostly in two hotspot positions, mapped at –124bp and –146bp upstream from the ATG start site. These two hotspots were reported to confer enhanced *hTERT* promoter activity, generating a consensus binding site (GGAA) for ETS transcription factors within the *hTERT* promoter region in melanoma (Horn et al. 2013).

Interestingly, we found four cases with mutations in the *EGFR* gene, three of which also had *BRAF* mutation: one case harbored the most common pathogenic variant p.L858R, which was found to be annotated in the COSMIC database related not only for lung carcinomas but even to six thyroid tissues of origin. The other two *EGFR* variants (p.A859T and p.R705K) were annotated in COSMIC database for lung carcinoma and urinary tract and were both considered probably damaging

by PolyPhen. Previous studies have failed to identify the *EGFR*-activating mutations in thyroid carcinomas (only 3.2% mutated cases) by direct sequencing (Mitsiades et al. 2006, Lee et al. 2007); however, Masago and coworkers (Masago et al. 2009) reported 7 positive cases out of 23 primary PTCs for exons 19 and 21 using more sensitive techniques (PNA-LNA clump).

Among these seven positive cases of Masago's series (Masago et al. 2009), three were reported having concurrent *BRAF* mutation and one patient with PTC whose tumor harbored the *EGFR*-activating mutation, showed a marked response to an *EGFR*-tyrosine-kinase inhibitor (Gefitinib).

In this study, we did not find any pathogenic variants of *KRAS*, *NRAS* and *HRAS*. As previously described, *RAS* mutations are more prevalent and seem to be more relevant as a prognostic indicator in follicular lesions than those in classic PTC (Sobrinho-Simoes et al. 2008, Giordano et al. 2014).

To date, tyrosine kinase inhibitors (TKIs) represent a new approach to the treatment of advanced cases of RAI-R DTC. Recently, sorafenib (ClinicalTrials.gov identifier NCT00984282) and lenvatinib (ClinicalTrials.gov identifier NCT01321554) have been approved for DTC. Vemurafenib (ClinicalTrials.gov identifier NCT01286753) is studied for the treatment of RAI-R, progressive, metastatic or unresectable PTC patients with *BRAF* p.V600 mutation with a good progression-free survival in both cohort of patients (TKI treatment naïve or TKI treated) (Brose et al. 2014), data recently confirmed by Dadu and coworkers (Dadu et al. 2015). Pazopanib, which targets VEGFR, PDGFR, c-KIT and other kinases, is studied in a cohort of RAI-R and rapidly progressing metastatic DTC (ClinicalTrials.gov identifier NCT00625846), with a good partial response (Bible et al. 2010). The future steps in treatment of advanced thyroid cancers should be targeting different molecular pathways and combined therapies to obtain a synergistic effect to control the disease and to reduce drug toxicities and side effects.

In conclusion, despite a significant prevalence of *BRAF* mutation, more than 70% of HPTCs in our series showed concurrent mutations of other genes such as *TP53*, *PIK3CA*, *CTNNB1* and *hTERT*, in contrast to classic PTC. The molecular portrait of HPTC reported in the present study further outlines the complex pathogenetic mechanisms of this variant and may offer the tumor genotype of HPTC to direct new strategies for a personalized treatment of these tumors.

Declaration of interest

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

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Author contribution statement

Luca Morandi designed the experiments, performed mutational analysis and wrote the manuscript; Leonardo Caporali and Alberto Righi performed immunohistochemical and mutational analyses; Francesca Maletta, Fabio Pagni, Sabrina Rossi and Anna Sapino collected HPTC cases and did the revision of histological preparations; Marco Gallo recruited clinical and follow-up data of the HPTC patients; Paola Rucci did statistical analysis; Riccardo V Lloyd and Sofia Ascoli coordinated the research group, they performed the revision of histological preparations, the supervision of molecular analysis and they wrote the manuscript.

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