Dear Editor,

Well-differentiated papillary thyroid carcinoma (PTC) accounts for ~90% of all thyroid cancers. While the majority of these tumors tend to behave as indolent lesions, a fraction of PTCs is highly aggressive and results in disseminated systemic spread to distant sites (Pellegriti et al. 2013). Numerous studies attempted to define the prognostic markers able to discriminate at diagnosis PTCs with more aggressive behavior from those with an indolent course (Handkiewicz-Junak et al. 2010). Nonetheless, the usefulness of genetic analysis in PTC patient management is still controversial, probably due to the fact that our knowledge about the genetic asset of aggressive PTCs is still very limited. The low occurrence of distant metastases and death among the overall PTC cases, and hence the difficulty in collecting large cohorts of PTCs that developed distant metastases (DM-PTCs), has been an important limitation for studies that attempted to correlate genetic alterations with prognosis and outcome of PTC patients.

An additional level of complexity on this issue comes from the genetic heterogeneity of tumors. Mutations that are drivers of aggressiveness are likely to occur as late secondary events during tumor progression and to be confined to small sub-populations of cells, which may compromise the possibility of detecting them using the standard sequencing technique.

Based on this consideration, we investigated a large cohort of 39 DM-PTCs and 20 control PTCs (without distant metastases), by performing a deep sequencing analysis of eight mutational hotspots in KIT, MET, PIK3CA, and PTEN genes. These hotspots were chosen as they were associated with aggressive features of various epithelial cancers, but they have been scarcely investigated or rarely found mutated in the general population of non-aggressive well-differentiated PTCs (Garcia-Rostan et al. 2005, Wasenius et al. 2005, Hou et al. 2007, Broecker-Preuss et al. 2008, Santarpia et al. 2008, Xing 2010).

Next-generation sequencing analysis of these hotspots (KIT exons 9, 11, and 13; MET exons 2 and 14; PIK3CA exons 10 and 21, and PTEN exon 5) was performed on FFPE-extracted DNA using the 454 GS-Junior Next Generation sequencer (Roche Diagnostics), with the cut-off of 5% mutated read percentage. A total of 79 mutations were detected in the overall cohort of samples. All mutations were single nucleotide variants. The number of total alterations detected in the DM-PTCs was strikingly higher than in the control group. Indeed, 76 mutations were detected in the DM-PTCs, while only five were observed in the control PTCs. Two mutations were found in both groups (Fig. 1A). Of the 76 mutations found in DM-PTCs, 64 were non-synonymous (NS), including five stop-codon mutations and 59 missense mutations. Among the 64 NS mutations, 22 were previously reported in databases (COSMIC and Ensembl) or publications, and 42 were not described. Of the five mutations found in the control PTC group, four were missense, including the two mutations shared with the DM-PTCs (METp.E168D and PIK3CAp.R524K). One of the four NS mutations was a novel mutation. Most mutations were found in a single sample, and no mutation was found in more than four samples.

As well, the number of mutational events was significantly higher in DM-PTCs than in control PTCs, with 82 events in DM-PTCs vs nine events in control PTCs (2.10 ± 2.95 vs 0.45 ± 0.60 events per sample respectively; P = 0.02). Within the control PTC group, 12 tumors developed lymph node metastasis while eight did not form metastases at any site and were confined to the thyroid. We did not observe any difference in the number of mutational events between these two subgroups (0.5 ± 0.5 vs 0.35 ± 0.74 event per sample respectively; NS).

Figure 1B depicts the number and frequencies of PTCs with at least one NS mutation in the DM-PTC and in the
### Gene mutations in PTCs with distant metastases

#### A

- **DM-PTCs**: Total mutations = n=76, Non-synonymous mutations = n=64, Stop mutations = n=5, Shared mutations = n=2, Non-synonymous mutations = n=4.
- **Control PTCs**

#### B

- **59 PTCs**
  - 23 DM-PTCs with NS mutations (0.59)
  - 16 DM-PTCs with NS mutations (0.41)
  - 7 Control-PTCs with NS mutations (0.35)
  - 13 Control-PTCs w/o NS mutations (0.65)
- **DM-PTCs**: MET (15 NS-mutations) 8 mutated DM-PTCs (0.5), PIK3CA (9 NS-mutations) 12 mutated DM-PTCs (0.81), PTEN (12 NS-mutations) 7 mutated DM-PTCs (0.19).
- **Control PTCs**

#### C

- **Number of NS mutations**

#### D

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Type</th>
<th>n Samples</th>
<th>Mutated read %</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIT</td>
<td>exon 9</td>
<td>M 1 9</td>
<td>15.3</td>
</tr>
<tr>
<td>BRAF</td>
<td>exon 14</td>
<td>M 1 11</td>
<td>15.3</td>
</tr>
<tr>
<td>PTEN</td>
<td>exon 10</td>
<td>M 1 13</td>
<td>15.3</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>exon 21</td>
<td>M 1 11</td>
<td>15.3</td>
</tr>
</tbody>
</table>

#### E

- **Mutated allele %**

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control PTC groups, and the distribution of NS mutations in the four genes analyzed. Twenty-three DM-PTCs (0.59) showed at least one NS mutation in the target gene regions analyzed. Nine DM-PTCs (0.23) showed only one mutation, while 14 DM-PTCs (0.36) presented more than one mutation, up to 13 different mutations (Fig. 1C, continuous red line). In the control PTC group, seven out of 20 PTCs showed at least one NS mutation (0.35), of which six PTCs showed only one mutation (0.30) and one PTC harbored two mutations (0.05) (Fig. 1C, dashed blue line). The average number of NS mutations per sample was 1.77 ± 2.59 in DM-PTCs vs 0.40 ± 0.60 in control PTCs (P = 0.02).

The complete list of the detected mutations is reported in Fig. 1D.

The percentage of mutated reads of NS mutations ranged from 5 to 69.7% in the DM-PTCs and from 22.9 to 47.9% in control PTCs. The average mutated allele percentage was significantly lower in DM-PTCs than in the control group (12.32 ± 10.39 vs 35.29 ± 11.37%; P < 0.0001). The read percentage of the NS mutations in the four genes in each DM-PTC and control PTC is shown in Fig. 1E. The PIK3Cap.G1049S mutation showed an allele percentage above 50% (69.7%) in one DM-PTC, suggesting the duplication of the mutated allele in tumor cells harboring these mutations.

Overall, in DM-PTCs, we observed a high number of NS mutations at low allele percentage (mostly between 5 and 10%), whereas, in control PTCs, few NS mutations were found, generally at higher allele percentage. This is in agreement with the hypothesis that tumor progression relies on progressive accumulation of genetic alterations.

As the BRAF V600E mutation is the most common genetic alteration in PTCs, with a frequency of ~50% among all cases, we investigated the relationship between the occurrence and allele percentage of KIT, MET, PIK3CA and PTEN mutations and BRAF V600E mutation (as determined in Sancisi et al. (2012) and Gandolfi et al. (2013)). We did not observe any differences in the total number of alterations in the four analyzed genes between BRAF-mutated and WT DM-PTCs (2.64 mutations on average in V600E-mutated DM-PTCs vs 1.28 mutations in BRAF-WT DM-PTCs; P = NS). Furthermore, we did not observe any correlation between BRAF status and occurrence of mutations in each of the four genes. In general, the V600E allele percentage tended to be higher than the allele percentage of mutations found in the KIT, MET, PIK3CA, and PTEN genes in the same sample (Fig. 1E), suggesting that BRAF V600E is acquired earlier during cancer development.

This work shows that the minority of clinically aggressive PTCs that develop distant metastases tend to accumulate a high number of mutations in the MET, KIT, PIK3CA, and PTEN genes, known to be essential for tumor maintenance and progression in a variety of other human cancers. MET was the gene with the greatest number of mutations, and ~36% of DM-PTCs and 30% of all PTCs harbored mutations in this gene. Approximately 30% of the DM-PTCs harbored mutations in more than one of the analyzed genes. The coexistence of genetic alterations in different PI3K/Akt and MAPK pathway genes has been observed rarely in differentiated thyroid tumors but frequently in anaplastic carcinoma (Hou et al. 2007, Xing 2010).

In DM-PTCs, more than 80% of the mutations were found at a low frequency and restricted to small subclonal populations within the tumor, suggesting that they occurred late during tumor development. In most cases, different mutations within the same PTC sample were at different mutated allele percentages, thus they were probably present in separated cell sub-populations.

The evolution of tumors is a process that involves the stepwise acquisition of multiple malignant features, and most of these changes are gained by genetic alterations on key genes. Our data are in agreement with this model, as DM-PTCs displayed a higher rate of mutation and a higher level of genetic heterogeneity when compared with control tumors.

The unexpected genetic complexity of tumors revealed in latest years by the advent of new sequencing
technologies seems to underline the inadequacy of the approach used until now, focused on the identification of single molecular markers as major determinants for tumor prognostication. In order to improve our understanding of the type and pattern of genetic alterations that are relevant to define the prognosis of patients with PTC, it is crucial to collect and analyze in depth large cohorts of those uncommon thyroid carcinomas that behave aggressively.

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

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Author contribution statement
G Gandolfi, D de Biase, A Pession, G Tallini, and A Ciarrocchi conceived the study and carried out experiments. S Piana and M Ragazzi retrieved the cases and performed histological analysis. D de Biase and G Acquaviva performed next-generation sequencing experiment and analyzed the data. G Gandolfi and V Sancisi performed the statistical analysis. G Gandolfi and A Ciarrocchi wrote the manuscript. All authors reviewed and edited the manuscript.

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