Author's response to reviews

Title: A mutation screening of oncogenes, tumor suppressor gene TP53 and nuclear encoded mitochondrial complex I genes in oncocytic thyroid tumors

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Point-by-Point reply to reviewers

Reviewer 1:

In the manuscript by "A mutation screening of oncogenes and nuclear encoded mitochondrial complex I genes in oncocytic thyroid cancer", Giovanni Romeo's group has studies the mutation profiles of a panel of 45 thyroid tumors by performing a candidate approaches. On the overall this is an interesting and important contribution to the field of thyroid tumors. The quasi- absence of detection of p53 mutation together with the finding of mutations in mitochondria function are interesting per se and should be published. I do not have any specific concerns. It would may be interesting to replace this study in the light of the large deep-sequencing effort. Is there any thyroid tumors that was fully sequenced and does it match with this targeted approach?

To our knowledge, no studies have been already published on massive sequencing analysis of oncocytic thyroid cancers in terms of exome/genomes; several groups have performed targeted analyses using massive omics technologies on different type of thyroid tumors, including small groups of oncocytic ones, and findings similar to our data were identified. We cited these studies in the text, as references #24 (Ganly et al, 2013) and #28 (Nikiforova et al, 2013).

I think it would make sense also to change the title as the title states about oncogenes but no tumor suppressor genes while the interesting data are found with p53 (that is a tumor suppressor gene).
This is a very useful suggestion and we have now modified the title accordingly:
A mutation screening of oncogenes, tumor suppressor gene TP53 and nuclear encoded mitochondrial complex I genes in oncocytic thyroid tumors

Reviewer 2

Evangelisti et al. report in thyroid oncocytomas on genetic alterations of nuclear encoded complex I genes and on genes typically altered in thyroid cancers. As oncocytic thyroid cancers have not been investigated regarding these genetic changes the manuscript sheds light on the genetics of these rare tumors. The most interesting finding was the occurrence of TP53 mutations in 3 out of 45 cases and of PAX8/PPARγ rearrangement in 5 out of 10 investigated samples.

Major Compulsory Revisions

A detailed supplementary table has to be prepared listing all samples grouped according to subtypes (hyperplastic oncocytic thyroid nodules, thyroid oncocytic adenoma, carcinoma, thyroid follicular adenoma) and detected genetic changes (nuclear and mtDNA), histology, age of patients and gender.

We thank the reviewer for the suggestion and we modified the text introducing a novel Supplementary Table 1, with tumor subtypes, age, gender, and nuclear and mtDNA mutations identified.

Discussion: In line 314 the authors claim that the expression of the chimeric protein PPFP did not show any significant correlation with the presence of mtDNA mutations. However, the reviewer could not find any data on the expression of the chimeric protein in the methods and results section. The authors need to include the data and method.

We are sorry that this phrase was misleading, because we did not want to indicate that we measured the expression level of PPFP protein (which actually we did not), but just reporting that the rearrangement, known in literature to produce the PPFP protein, did not correlate with the presence of mtDNA mutations. We have now removed the mention to PPFP protein in the text.

As the frequency of TP53 mutations in the samples is very low (3 out of 45) and the incidence of mtDNA mutations is about 50% in the samples the co-occurrence of mtDNA mutations and TP53 mutations in two cases are more likely a coincidence, unless statistical analysis would indicate a clear cut correlation. Therefore, the speculation on the impact of TP53 mutations on the occurrence of the oncocytic phenotype is more than questionable and therefore, the emphasis given to this correlation in the abstract should be omitted and in the discussion should be even more speculative.

We modified the text accordingly in order to avoid any emphasis on this issue, in the abstract as follows: “In our oncocytic tumor samples we identified rare TP53 mutations” (page 3, line 68). In the Discussion: “Tumor suppressor p53 has been largely implicated in the metabolic remodeling that cancer cells develop during progression, particularly through the regulation of mitochondrial respiration via
TIGAR and COXIV of the respiratory chain [29]. Nevertheless, several studies have shown that in thyroid oncocytic tumors a burden of mtDNA mutations all impinging on the bioenergetics competence of thyroid cells may give rise to an aberrant mitochondria-centered compensatory mechanisms and ultimately to the oncocytic phenotype [14]” (Pages 14-15, lines 340-347).

The message of the last sentence of the discussion is not clear the reviewer.

We modified the text as follows: “This indicates that other genomic alterations may induce metabolic microenvironment changes drivers of tumorigenesis, coupled to mitochondrial abnormalities [13, 30]” (Page 15, lines 352-354).

Conclusion: It is not obvious from the results that a significant co-occurrences of genetic effects have been found in the oncocytic tumors. This might get clearer once a table including all cases and genetic alterations is included.

We have now reported all the changes identified for each included sample in Supplementary Table1.

Minor Essential Revisions

The exact method of DNA/RNA extraction should be given, as the extraction of high quality RNA is difficult from paraffin embedded tissues. This should include the amount of tissue used for the extraction as well as the quality measures taken.

We inserted the details in the Method section: “Total RNA was extracted using the RecoverAll kit (Ambion Inc., Austin, Texas, USA) starting from four 20-µm-thick slides, in accordance to the manufacturer’s instructions. RNA concentration was measured using Quant-itTM RNA kit” (page 8, lines 176-178). As index of RNA quality the beta-actin gene was used as internal control. This method has been reported in reference #17.

In the text the authors indicate TP53 mutations in 3 cases but in table 3 only two cases with frameshifts are mentioned. The authors need to include the missense mutation in the table.

We apologize for the mistake, we have amended Table 3.

Age range and gender should be given of the sample subgroups.

These informations (when available) are now presented in Supplementary Table 1.

The full name of genes/protein should be given of all abbreviations used when first mentioned (e.g. RET, PCT, PAX8, PPARg).

We specified the full name of the genes “B-Raf proto-oncogene (BRAF), Harvey rat sarcoma viral oncogene homolog (H-RAS), Neuroblastoma RAS viral oncogene homolog (N-RAS), and Kirsten rat sarcoma viral oncogene homolog (K-RAS), the fusion genes REarranged during Transfection (RET)/PTC1,
RET/PTC3, Paired Box 8 (PAX8)/peroxisome proliferator-activated receptor gamma (PPAR##, and Tumor Protein p53 (TP53)” in the Introduction (page 8, lines 142-146).

Methods:
Line 149: change to „.....was performed“
Corrected (now page 152).

As no reference is given for the methods of BRAFp600V>E and RAS codon 61 mutations it is necessary that primer sequences for PCR amplification are given.

We have now inserted the corresponding reference, reference #16 (Piana et al, 2013).

Line 286: Oncocityc should be Oncocytic
Corrected, thank you.

Line 292: Include a space after the end of the sentence.
Corrected, thank you.

Discretionary Revisions
The number of samples analyzed should be indicated in the abstract.

We inserted it (“45”; page 3, line 62).

Line 103: change the time to „.....cells originate“.
Corrected (page 5, line 102)

We have specified it throughout the text.

Line 125, 130, 285: The authors should also refer to some publications reporting primary data on mtDNA mutations in different oncocytomas and the functional impairment of complex I (eg. Hum Mol Genet. 2008 Apr 1;17(7):986-95)

We modified the text accordingly: “albeit it has to be noted that in other organs, such as kidney and pituitary gland, the correlation between the occurrence of such mutations, the oncocytic phenotype and the functional disruption of complex I activity is far more stringent than in the thyroid [6], [10], [11], [12], [13]. Thyroid tumors may present as heterogeneous neoplasms, in which oncocytic cells are more or less a predominant component, and heterogeneity of nuclear and mitochondrial gene lesions may be envisioned [5], [14] (Introduction, Page 6, line 125-132), and in the Discussion as follows: “the co-occurrence of mtDNA alterations, the oncocytic phenotype, and a heavy dysfunction in the oxidative phosphorylation (OXPHOS) complexes activity, in particular in complex I [4, 13]. The strength of a correlation between mtDNA mutations and functional
impairment of complex I is even more striking in other oncocytomas, e.g. renal and pituitary oncocytic tumors [11], [12], [13], [14].