

Reviewer 2

The article provides a clear and detailed analysis of the relationship between MITF expression and several genetic and clinical parameters in UM. The use of tables and figures also help in interrupting the results.

My questions: Please provide detail

Reply: We thank the reviewer for the thoughtful comments and will do our best to address each question, with the knowledge we have at the moment. The line numbers mentioned in the reply refer to the manuscript version with track changes.

Any inverse relationship suggest a potential therapeutic target for UM treatment?

Reply: In the field of uveal melanoma, we are always in search of new targets that allow us to stop or decrease the metastatic burden. MITF is an attractive target, but one should be very careful because of its many functions in different cells and different cell states. Our results show that tumours with low MITF have more malignant features than tumours with high MITF, but evidence from cutaneous melanoma shows that MITF-high cells, while being less invasive, are highly proliferative. Moreover, there are contrasting reports on the role of MITF in the responsiveness or resistance to existing therapies. We believe that more studies are needed to pinpoint the most efficient way to target the MITF pathway and we do not wish to speculate too much. We did not any speculation to the text.

What is the relationship between MITF expression levels and survival in patients with UM, inflammatory phenotype or genetic affect this relationship?

Reply: We believe that MITF is involved in tumour behaviour but not independently. In our cohort, as in the study by Mouriaux et al., we did not find a significant difference in survival between UM with low and high MITF. However, a small group of 10 cases with very high MITF expression had excellent prognosis, when compared to the rest of the cases. Our data also show that 9 of these 10 cases have disomy 3. MITF expression is higher in disomy 3 than in monosomy 3 tumours and shows an inverse correlation with inflammatory markers. We believe that all these factors have a role and influence each other, with monosomy 3 being the chief driver of a poor prognosis and a prominent inflammatory microenvironment. We cannot say at this stage if MITF directly influences the expression of inflammatory markers or if the reverse is true. However, evidence from studies on cutaneous melanoma shows an inverse relation between MITF and inflammatory markers and that modulation of MITF expression causes changes in the inflammatory response [1-3]. These observations suggest that the modulation of MITF expression in uveal melanoma cells may be part of the process of tumour progression and metastasis formation, and one of the pathogenic routes following a BAP1 mutation/Chrom 3 loss.

We have now extensively discussed these concepts in the discussion section (lines 459-498).

What implications do these findings have for the understanding of the relationship between MITF and pigmentation in UM?

Reply: MITF has many functions and targets in melanoblasts, melanocytes and melanoma cells, only some of which are related to melanin synthesis and pigmentation. In a recent review, we explored the previous literature regarding the role of MITF in melanocytes, cutaneous melanoma and uveal melanoma and the complex relationship between MITF and cellular processes such as cell survival, proliferation and invasion [4]. We found a negative correlation between MITF expression (both mRNA and protein) and pigmentation (and pigment-related genes). This suggests that in UM, the non-pigment-related function of MITF may be more relevant than its pigment-related functions.

We added a section in the discussion regarding this. (lines 359-361)

What is the relationship between MITF expression and EMT and stem cell markers in the cohort of UM patients?

Reply: Previous studies in cutaneous melanoma showed that melanoma cells with high MITF have high TWIST1 and ZEB1 and are more invasive and less proliferative, while melanoma cells with low MITF have high ZEB2 and SNAI2 and are more proliferative and less invasive. A study by Asnaghi in UM corroborated this model in UM as well, with high TWIST1 being related to a poor prognosis. We now show that MITF expression levels are indeed negatively correlated to TWIST1 and positively correlated to SNAI2 and ZEB2, thus further corroborating the similarity with cutaneous melanoma. We found a positive correlation between BAP1 and SNAI2 expression and a negative correlation between BAP1 and TWIST1 expression (data not shown in the manuscript). These findings prompted us to postulate that BAP1 inactivation may be driving this EMT shift, possibly through MITF. Since these are *in silico* observations, we will be curious to see if this hypothesis can be confirmed in functional tests. This will be future work. These concepts are extensively explained in the discussion section of the manuscript (lines 499-525).

What is the significance of the downregulated genes IRS2, AHCYL2, PPP1R3C, SNHG7, and CDV3 in UM with low MITF expression?

Reply: We have now updated the manuscript by moving gene descriptions to the discussion section. We hope that the new part in the discussion regarding clarifies their potential significance. IRS2 and PPP1R3C are involved in glycogen synthesis. Since GSEA showed that the metabolic pathways GLYCOLYSIS, OXIDATIVE_PHOSPHORYLATION and KRAS_SIGNALING_UP and MTORC1_SIGNALING were enriched in MITF-low UM, we think tumours with low MITF may have a different metabolic landscape when compared to MITF-high UM. AHCYL2 and SNHG7 have been linked to proliferation arrest and cell cycle arrest (SNHG7 in UM cell lines specifically). Therefore, the downregulation of these two genes may be needed for the malignant progression of UM. A more detailed discussion of this topic can be found in the discussion section of the manuscript (lines 410-444).

What is the significance of the enriched pathways in UM with low MITF, particularly those related to immune processes and metabolic reprogramming, and how do they relate to MITF expression?

Reply: As discussed in the answer to the second question, previous studies have shown that MITF and the inflammatory response are related in cutaneous melanoma, with inflammatory markers influencing pigmentation and MITF expression and with modulation of MITF expression causing changes in the inflammatory response [1-3]. The fact that the most enriched pathways in MITF-low UM are related to immune processes lends support to these studies. A more prominent inflammatory infiltrate has been shown to be related to a worse prognosis in UM. We do not know if changes in MITF expression directly influence inflammatory markers in UM or if both phenomena occur as a consequence of a common upstream process related to loss of BAP1/chromosome 3. Nevertheless, we can hypothesise that, although not directly related to patient survival, MITF has a role in UM behaviour and that the modulation of its expression in UM cells may be part of the process of tumour progression and metastasis formation. (lines 459-498).

As for the pathways involved in metabolic reprogramming, the role of MITF in metabolism has not been extensively studied in the literature. Some studies have shown an association between MITF and hypoxia and have postulated that hypoxia and nutrient starvation may cause a decrease in MITF and greater invasiveness through HIF1 α [5-7]. However, the HYPOXIA pathway is not differentially enriched in MITF-low vs MITF-high UM in our cohort. Haq et al. reported that activation of BRAF/MAPK pathway in cutaneous melanoma cells decreases MITF, which in turn decreases PGC1 α , which is a potent activator of oxidative phosphorylation [8]. If a similar mechanism, possibly initiated by BAP1 inactivation, is present in UM as well, it may partially explain the different behaviour of tumours with high and low MITF. This point is discussed in the discussion section, lines 429-444.

Additionally, why were EMT factors not among the most differentially expressed genes in the analysis and why was the Hallmark EPITHELIAL_MESENCHYMAL_TRANSITION pathway not significant?

Reply: EMT is an extremely complex process, and different factors have different and sometimes contrasting roles in different types of cancers, as extensively explained in the review by Nieto et al (Nieto, M.A., Huang, R.Y., Jackson, R.A., Thiery, J.P., 2016. EMT: 2016. Cell 166, 21-45.). The work of Caramel, Deckener and Vandamme showed that in cutaneous melanoma, only a subset of EMT markers (ZEB1 and TWIST1) are related to invasion while other markers (ZEB2 and SNAI2) act as tumour suppressors and are positively related to MITF. Therefore, the EMT process is extremely heterogeneous and the Hallmark pathway EPITHELIAL_MESENCHYMAL_TRANSITION does not distinguish between specific subsets of EMT markers. This may be the reason why EPITHELIAL_MESENCHYMAL_TRANSITION is not significantly enriched in MITF-low tumours. As for the lack of EMT markers among the most differentially expressed genes, we need to take into account the strict cut-offs of differential gene expression analysis, which uses FDR and multiple testing correction. While it is useful to identify genes that show highly significant differences between the two groups, it may dismiss genes with lower significance that may be part of important processes.

We did not extend the discussion on this subject as it already has quite a long text about this, and it would be quite speculative.

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