Author’s response to reviews

Title: Constitutional mutation in CDKN2A is associated with long term survivorship in multiple myeloma

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Author’s response to reviews:

Dear Editor,

We would like to thank yourself and the reviewer for your interest in our manuscript and helpful comments. Please find below detailed comments to the reviewer’s queries. We have addressed all issues raised and provide clarifications to some sections. We hope with these improvements you will find our manuscript suitable for publication in BMC Cancer.

Yours faithfully,

Vallari Shah, MD

Reviewer reports:

Sabarinath Venniyil Radhakrishnan (Reviewer 1): In this case report "Constitutional mutation in CDKN2A is associated with long term survivorship in multiple myeloma: a case report" Shah et al has described a patient who developed myeloma at a young age and subsequently also had
multiple melanomas and other tumors and was found to have a germline mutation in CDKN2A. The case presentation is interesting but the work up is incomplete in my opinion. Please see my comments below

1) The authors have not described how the genetic testing was done and what the allele frequency was?

Genomic DNA PCR amplification using primers described previously of the 4 exons of CDKN2A (exons 1α, 1β, 2 and 3) (Harland M et al, European Journal of Cancer 2008) was performed. PCR fragments were isolated by agarose gel electrophoresis and purified prior to Sanger sequencing using QIAquick Gel Extraction Kit (Qiagen, Paisley, UK). The allele frequency of the c.213C>A mutation was 1 with a heterozygous mutation noted. This is shown in Figure 2 of the Sanger sequence chromatogram from the patient showing both the C and A base at position 213. We have now included these additional details in the full text of the article (Case Presentation, paragraph 5, line 10).

2) I understand that the bone marrow for MM was done 30 years back and so would not be possible to have genetic studies done on the plasma cells but she has recent lung cancer and also multiple melanomas but no mention of genetic abnormalities in these tumors. It would be very interesting to know if there is a loss of heterozygosity of these this locus in these tumors. Furthermore, there is no family history of melanoma or pancreatic cancer as described in the report and so it would be speculative to assume this mutation is associated with myeloma.

In response to the reviewer’s comment we have now sequenced tumour DNA extracted from the patient’s lung cancer tissue. We detected loss of heterozygosity at the same base where the heterozygous mutation (c.213C>A) was seen in the germline DNA (See Chromatogram below). This has been included in Figure 2 as well as discussed in the Case Presentation, Paragraph 6, Line 8.

In terms of family members, the patient’s son at the age of 34 was diagnosed with melanoma which was excised. The patient’s son did not wish to undergo further genetic testing due to potential issues with regards to health insurance. This is mentioned briefly in paragraph 5 of the case presentation. This is indicative but not conclusive of the pathogenic nature of the mutation.
We have provided further evidence of the pathogenicity of this lesion in response to the reviewer’s third question.

3) Please provide evidence of the pathogenic nature of this missense mutation as reported on line 54. The previous report of germline mutation in CDKN2A associated with MM is not a missense mutation.

Evidence of the pathogenic nature of the mutation is as follows:

1. Family studies: A study by Soufir et al (Hum Mol Genet, 1998) carried out mutational analysis on 40 melanoma prone families. The c.213C>A mutation leading to the Asn71Lys amino acid change was identified in one of these families and was shown to segregate with disease (3 cases). Another study by Bishop et al (JNCI 2002) which analysed 80 families with inherited susceptibility to melanoma found 1 family carrying the c.213C>A missense mutation within the CDKN2A gene. Goldstein et al (J Med Genet, 2007) listed the same c.213C>A mutation in 2 European melanoma families.

2. Functional Assays: Functional assays of INK4A function were measured by McKenzie et al (Hum Mut, 2010). In vivo binding activity of all p16 was assessed using a mammalian two hybrid assay. Any variant with <85% wild type CDK4 of CDK6 binding affinity was classed as deleterious. The Asn71Lys variant had impaired binding to CDK4 and CDK6 (7.3% and 11.7% respectively; wild type p16 has 100% binding affinity). The subcellular localisation of each p16 construct was evaluated after transient transfection of NM39 cells. The Asn71Lys mutant did not have wild type localisation.

3. In Silico Predictions: To predict the consequences of missense variants in silico, we have tested 3 different algorithms (Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and mutation taster (http://www.mutationtaster.org/). The majority of these algorithms predict that this is a pathogenic mutation. Specifically, all 3 algorithms predicted the Asn71Lys amino acid substitution in the INK4A protein to be pathogenic (“Probably Damaging” with a score of 0.992 by PolyPhen-2, “Damaging” by SIFT with a score of 0.03 and “Disease Causing” with a score of 94 by Mutation Taster).
4. We also searched ExAC (Exome Aggregation Consortium) database and did not find the c.213C>A mutation listed, indicating that this is not a normal variant.

5. The c.213C>A missense mutation has also been found in a patient with a supraglottic SCC (T2N2aM0) as described by Fischer et al. (Eur Arch Otorhinolaryngol, 2007) and has been deposited within the Cosmic database.

6. Additionally, evidence for the association of the CDKN2A gene and its association with myeloma susceptibility has been shown in genome wide association studies which found a susceptibility locus for myeloma at chromosome 9p21.3 variant rs2811710 of CDKN2A (Mitchell JS et al., Nature Communications 2016). A population based study in 1354 people with multiple myeloma in Utah also suggests a link between multiple myeloma, melanoma within first and second degree relatives (Camp NJ et al., New England Journal of Medicine, 2008). This has been further confirmed in other studies (Altieri A et al., European Journal of Cancer, 1990; Kristinsson SY et al., Haematologica, 2009; Lynch HT et al., JNCI, 2001).

These have been included in Paragraph 1 and Paragraph 3, Line 4 of Discussion and Conclusions Section.

Douglas Sborov (Reviewer 2)

Shah et al present a patient with MM that had a profound response to melphalan with sustained complete response for over 30 years. Development of other malignancies led to investigation for a germline CDKN2A mutation, and the authors argue that this germline mutation may be associated not only with development of MM, but also with profoundly chemo-sensitive disease.

Page2 Line42 - Statement "subsequent introduction of IMiDs, PIs, and autoHSCT" is dated, and would be strengthened by mention of 1) maintenance therapy, 2) new therapies including monoclonal antibodies and HDACIs, and 3) references to recent trials supporting the use of autoHSCT/MEL in the era of novel therapeutics.
We have added the above comments within the manuscript and referenced trials as suggested above. Paragraph 1 of background, Line 7.

Page2 Line45 - The statement "significant variation in outcome" would be strengthened by a very brief discussion of R-ISS staging and associated median OS rates as a means to identify those patients that we currently think have a possibility of long term remission.

We have included a discussion on the various staging systems within myeloma including R-ISS. Background section, Paragraph 2, Line 2.

Page3 Line12 - If available, can you provide the percentage of PCs in the initial biopsy?

Unfortunately, this is not available as it was done at another hospital and we have been unable to source the report. The patient underwent a bone marrow biopsy after 1 cycles of melphalan therapy which showed 7% plasma cells, prior to transplantation the patient had <5% plasma cells.

Page3 Line 39 - Has a repeat bone marrow biopsy been completed to evaluate for MRD?

The patient’s last bone marrow biopsy was carried out 12 years ago. This showed complete remission (plasma cells <5%) with no detectable paraprotein and normal light chains consistent with complete remission. MRD analysis by both flow cytometry or VDJ rearrangement analysis was not available at the time as it is a relatively recent development in routine clinical practice.

Page4 Line6 - Wording needs to be fixed, "with by"

We have amended this.

Page4 Line18 - "described a (not an) MM"

We have amended this.

Page4 Line17 - 1st paragraph of discussion, a more thorough discussion is warranted here. In addition to the mention of the Dilworth et al, Blood, 2000 article, discussion of recent GWAS
studies and the association between PC dyscrasias and CDKN2A-related risk loci would be interesting.

We have included further discussion of GWAS studies showing susceptibility loci at the CDKN2A locus as well population studies detailing the association of melanoma and myeloma in first and 2nd degree relatives in the discussion and conclusions section, paragraph 3, line 4.

Page4 Line25 - 2nd paragraph of discussion. In the latter part of the paragraph, an abbreviated list of mechanisms is provided. A more detailed explanation is warranted, including how CDKN2A and related mutations are directly associated with myeloma (development and treatment).

We have included a discussion on mechanisms of increased cancer susceptibility with mutations in CDKN2A and associations with downstream effectors in myeloma. Discussion and Conclusion section, paragraph 4, line 6 + paragraph 5, line 2 + paragraph 7 (whole paragraph)

Page4 Line47 - Provide rationale for incorporation of the MDM2 inhibitor discussion; ie. how is this associated to the discussion about CDKN2A mutations. The link presented is ARF but it is not very clear how it's presently written.

We have provided a clearer explanation for this within the manuscript. Discussion and Conclusions Section, Paragraph 8, line 7.

This patient provides an extremely interesting case that supports the possibility that a CDKN2A germline mutation may be associated with development of MM, and potentially increased susceptibility to melphalan therapy. That being said, a more clear and potentially hypothesis driven explanation and discussion would strengthen the manuscript.