Author's response to reviews

Title: HNF1B polymorphism influence the prognosis of endometrial cancer patients: a cohort study

Authors:

Vincenzo Dario Mandato (dariomandato@virgilio.it)
Enrico Farnetti (enrico.farnetti@asmn.re.it)
Federica Torricelli (federica.torricelli@asmn.re.it)
Martino Abrate (martino.abrate@asmn.re.it)
Bruno Casali (bruno.casali@asmn.re.it)
Gino Ciarlini (gino.ciarlini@asmn.re.it)
Debora Pirillo (debora.pirillo@asmn.re.it)
Mariacarolina Gelli (mariacarolina.gelli@asmn.re.it)
Mario Grassi (mario.grassi@unipv.it)
Stefano Palomba (stefano.palomba@asmn.re.it)
Giovanni B La Sala (giovannibattista.lasala@asmn.re.it)

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Author's response to reviews: see over
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“HNF1B polymorphism influence the prognosis of endometrial cancer patients: a cohort study”

Authors:

Vincenzo Dario Mandato: dariomandato@virgilio.it
Enrico Farnetti: enrico.farnetti@asmn.re.it
Federica Torricelli: federica.torricelli@asmn.re.it
Martino Abrate: martino.abrate@asmn.re.it
Bruno Casali: bruno.casali@asmn.re.it
Gino Ciarlini: gino.ciarlini@asmn.re.it
Debora Pirillo: debora.pirillo@asmn.re.it
Maria Carolina Gelli: mariacarolina.gelli@asmn.re.it
Davide Nicoli: davide.nicoli@asmn.re.it
Mario Grassi: mario.grassi@unipv.it
Giovanni Battista La Sala: giovannibattista.lasala@asmn.re.it
Stefano Palomba: stefanopalomba@tin.it

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Author’s response to reviews: see over
**Reply to Reviewers**

**Reply to Reviewer 1 - Francisco Candido dos Reis**

We would thank to Reviewer for his comments and suggestions that we have carefully followed and incorporated in the revised version. We hope that the new manuscript could be considered sufficiently improved to be suitable for publication in the current form.

**Major Compulsory Revisions**

1. The introduction is generic. The authors should present a clear justification for the importance of investigating rs4430796 as prognostic factor in endometrial cancer.

   **Re:** The Background section was deeply revised clarifying the importance to study rs4430796 as prognostic factor in endometrial cancer.

2. The survival analysis was based only in OS. The results would be more meaningful if progression free survival was also evaluated.

   **Re:** Progression free survival was evaluated as suggested and reported in multivariate analysis (see revised Table 2). In the revised abstract and result section the new findings were added. Finally, in consideration of the further endpoint evaluated the title of the manuscript has been changed accordingly.

3. The purpose of the Cox proportional hazards model is to simultaneously explore the effects of several variables on survival time; therefore “univariate” Cox model is not informative. On the other hand, the “multivariate” model should include known prognostic factors like age, grade, and performance status.

   **Re:** After evaluation of your comment by our statistician, it was confirmed the correctness of the procedure to adjust data only for age (as reported in Table 2). In our study FIGO grading could be applied only in 162 patients so we preferred to exclude grade from multivariate analysis. Only endometrioid tumours (type I EC) can be classified into highly differentiated (G1), moderately differentiated (G2), or undifferentiated (G3) according to FIGO classification because FIGO grading excludes serous or clear cell carcinoma (type II EC). Finally, we did not report performance status of the patients because all included patients were already initially selected for “full surgery” and adjuvant therapy.

4. Cox proportional hazards model can deal with binary, grouped or continuous covariates. The covariates stage and adjuvant treatment should not be used as binary in the model.

   **Re:** Thanks for your suggestion that we followed. Table 2 was revised as suggested.

5. There were 8/57 stage III-IV in GG genotype group and 19/134 in the GA+AA group. According to the Figure 2 more than 90% of stage III-IV EC in the GA+AA group (chemoradiotherapy group) are alive at 5 years of follow up. It looks similar to early stage tumors. This result is not compatible with the current literature, expected 5-years survival for this group would be around 50%. These cases should be reviewed.

   **Re:** The 5 years survival of all patients (27/191) at stage III-IV was less than 90%. Only patients at stage III-IV in the GA+AA group presented a better survival than expected. Probably, A allele could be associated with a better prognosis. Below you will find the figure showing the overall survival according to the stage independently from genotype. In the Figure a clinical and statistical difference between groups was showed. Moreover, the stage III-IV group had a lightly higher 5-year survival (when compared to literature) probably for a selection bias due to the inclusion and exclusion criteria used for enroll an homogeneous study population.
6. The authors should write a paragraph containing the meanings of their results in the discussion.
Re: As suggested by Reviewer the Discussion section was revised focusing the clinical and academic importance of the results obtained.

7. The clinical implications of the results should be better explained.
Re: See previous reply.

Minor Essential Revisions
1. In the line 105 “experimental study” should be “observational study”.

Re: The word was changed as suggested.

2. Table 1: the proportions should be reported by group.
Re. The Table 1 was revised as requested.

3. Figures 1 and 2: should include the corresponding at risk tables.
Re: As suggested, risk tables were added in the revised Figures 1 and 2.

Discretionary Revisions
1. The sentence “Parametric data were expressed as median and range” does not make sense.
Re: The sentence was changed in “Associations between rs4430796 and clinical and pathological parameters were assessed by generalized linear models”.

2. The sentence “Significant statements referred P values of two-tailed tests that were less than 0.05” should be deleted.
Re: The sentence was deleted as suggested.
3. The authors state “In all EC patients a complete resection of the disease was obtained.” Was it possible for stage IV tumors?

Re: As before reported we selected only EC patients treated with a complete resection of the disease, including stage IV EC. This issue, as before reported, could introduce a bias selection.

4. The sentence “Based on present findings, we estimate a post-study sample size of about 500 patients…” should not be in the discussion.

Re: The sentence was deleted as suggested.

Reply to Reviewer 2 - Madhuri Koti

We would thank to Reviewer for his positive comment to our manuscript. We hope that the new manuscript revised according to Reviewer 1 and 3 could be considered improved and suitable for publication.

Reply to Reviewer 3 - Jeremy Squire

Reviewer's report:

HNF1B is a member of the homeodomain-containing superfamily of transcription factors. The protein has been shown to function in nephron development, and regulates development of the embryonic pancreas. Mutations in the HNF1B gene are associated with a spectrum of human diseases including diabetes, renal cysts, and diseases of the pancreas, liver and genital tract. Thus it is likely that HNF1B has a role in the development and function of these respective organs.

More recently, genome-wide association studies have linked DNA sequence variants of HNF1B to both an increased risk of prostate cancer, and a protective effect against type 2 diabetes. These recent reports further define the wide ranging role of HNF1B in human health and disease.

This study is the first to examine the prognostic impact of the rs4430796 germline DNA variants in endometrial cancer (EC). It is therefore important because of this and the recent GWAS studies in ovarian cancer drawing attention to HNF1B variants.

Re: We would thank to Reviewer for his comments and suggestions that we have carefully followed and incorporated in the revised version. We hope that the new manuscript could be considered sufficiently improved to be suitable for publication in the current form.

There are some major points that must be addressed before this study can be published:

1. There is virtually no consideration of the biology of the rs4430796 SNP on HNF1B transcription factor function and how the GG allele may be mechanistically involved in poor outcome. The authors should provide more background about how variations of this protein might be associated with poor outcome in EC.

Re: In the revised version we have underlined that no data were found in literature explaining the role of rs4430796 genotype on HNF1B function but some contradictory studies supposed that rs4430796 polymorphism could influence HNF1B gene expression. Spurdle et al. analyzed several lymphocyte-derived gene expression datasets and identified significant associations between rs4430796 genotype and HNF1B expression in individuals of European ancestry. In the same population, the RNA sequencing experiment suggested that HNF1B expression in patients with different rs4430796 genotypes presented a trend of increase in read depth with increasing number of G alleles. Furthermore, recent studies, not always concordant, analyzed the influence of HNF1B gene expression on chemosensitivity in ovarian cancer. In particular, some studies, observing that ovarian CCC characterized by HNF1B overexpression were often chemo-resistant, suggested a possible effect of the level of HNF1B on chemosensitivity. It was demonstrated that shRNA mediated downregulation of HNF1B sensitizes ovarian cancer cells to cisplatin-or paclitaxel-mediated cytotoxicity, through inverse regulation of HSulf1 expression. In 2014 was also proposed an
interesting mechanism of inhibition of cell death by HNF1B transcription factor: the study demonstrated that chemoresistance that characterizes CCC might be due to aberrant retention of the G2 checkpoint of cell cycle induced by HNF1B overexpression [28]. HNF1B might induce this aberrant retention through the up regulation of CHK1 kinase, CHK1 kinase plays a pivotal role in the G2 checkpoint.

2. The proposed interaction (page 10) of the genotypes in the post-surgical radio-chemotherapy subjects is confusingly presented and also lacks a biological mechanism. Presumably some role for DNA repair related to advantage of one isoform over the other is involved? The reader needs some guidance to understand what model is being proposed and whether it makes biological and clinical sense.

Re: As suggested by Reviewer, the Discussion section was revised including a potential biological mechanism. As also above detailed, we believe that G allele might be associated with a HNF1B overexpression and the HNF1B overexpression might cause an aberrant retention of the G2 checkpoint of cell cycle resulting in chemoresistance.

3. There is a real risk with such a small sample size that there is marked bias in the distribution of the GG genotypes that has led to false conclusions in this particular study group. The authors should better justify why this first study is representative and not going to cause others to embark on fruitless validation studies.

Re: As detailed in the revised Discussion section, we agree that current study has also important limitations. In particular, it might be underpowered because of the small cohort, and actual sample size might not be sufficient to detect a synergistic effect in a replicate study. However, despite the small sample size, the genotype distribution in our population comply with Hardy-Weinberg equilibrium indicating that identified significant association can be considered representative and not-biased by patient selection. Regarding to small sample size, we did not manage to recruit a sufficient number of EC patients with advanced disease to request chemo-radiotherapy because most EC is diagnosed at early stage, and chemo-radiotherapy is commonly delivered in less than 10% of subjects. Chemo-radiotherapy is generally administered to EC patients with positive lymph nodes (stage III) or distant metastases (stage IV). That population represented the 6-7% and 3% of EC patients, respectively of the overall EC population. In addition, the rate of 5-year survival for stage I disease is approximately 80-90%, for stage II, 70-80%, and for stages III and IV, 20-60%. Hence the mortality is also low. All these aspects was discussed in the revised version.

4. How would the biology of HNFIB likely relate to established pathways in EC such as AKT and ARID1A? If there are no established links there are likely informatics approaches that could be used to demonstrate a putative relationship. Such data may add to the argument that this protein could impact the molecular oncology of EC profoundly.

Re: As suggested, the manuscript has been revised adding a short paragraph about published data suggesting a potential link between HNFIB and AKT/ARID1A in EC pathogenesis and progression. In literature there are no established links between HNFIB pathway with ARID1A and AKT pathway. ARID1A is a tumor suppressor gene. Inactivating mutations of ARID1A and loss of its expression was found especially in endometrioid-derived tumors, including ovarian CCC, ovarian endometrioid carcinomas and EC [Guan B, Mao TL, Panuganti PK, Kuhn E, Kurman RJ, Maeda D, et al. Mutation and loss of expression of ARID1A in uterine low-grade endometrioid carcinoma. Am J Surg Pathol 2011;35:625-32. Wiegand KC, Lee AF, Al-Agha OM, Chow C, Kalloger SE, Scott DW, et al. Loss of BAF250a (ARID1A) is frequent in high-grade endometrial carcinomas. J Pathol 2011;224:328-33]. ARID1A mutations co-occur with mutations of PTEN and PIK3. Furthermore, PI3K pathway activity is regulated by ARID1A through phosphorylation of AKT [Liang H, Cheung LW, Li J, Ju Z, Yu S, Stemke-Hale K, et al. Wholeexome sequencing combined with functional genomics reveals novel candidate driver cancer genes in endometrial cancer. Genome Res 2012;22:2120-9]. Regulation of PI3K pathway by ARID1A further suggests that ARID1A plays an important role in the pathogenesis of type I EC. Both ARID1A and HNFIB play a role in the pathogenesis of ovarian CCC arose in endometriosis. ARID1A mutation is an early event in neoplastic transformation and it is present both in carcinoma and contiguous atypical endometriosis, whilst HNFIB only in the CCC tissue [Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med 2010;14:1532-43].