Peer Review File

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Review Comments:

1. At present, the prediction mechanism of TMB tumor immune response is not completely clear. It is generally believed that the higher the TMB is, the more new antigens will be produced, that is to say, the immunogenicity of the body will be increased, and the tumor specific T cells will recognize the new antigens and finally produce immune response. Is there any other potential mechanism?

Response: Thank you very much for your question. At present, as you said, the main mechanism for predicting the immunotherapeutic effect of tumor mutation burden may be: large numbers of mutations produce numerous altered peptides, of which a subset are successfully expressed and processed by the major histocompatibility complex, resulting in neoantigens to which the immune system can generate an antitumor response. Therefore, the more DNA mutations that exist, the more candidate peptides will be generated, resulting in a higher likelihood of neoantigens being successfully recognized by the immune system[1]. Naiyer A. Rizvi et al. validated the specificity of the neoantigen-reactive T cells, PBLs(peripheral blood lymphocytes) from days 63 and 297 were expanded in vitro in the presence of mutant peptide and subsequently restimulated with either mutant or wild-type peptide (ASNASSAAK versus ASNAPSAAK), and intracellular cytokines were analyzed. At both time points, a substantial population of polyfunctional CD8+ T cells was detected in response to mutant but not wild-type peptide. In addition, although TMB is often used as an immunotherapy biomarker, there is no evidence of a direct correlation between TMB and PD-L1 expression and the T-effector and IFN- γ gene signatures[2].

2.Based on KEGG and go analysis, we selected DEGs and used multiple regression models to further select 10 TMB related features. Have you further studied the predictive efficacy of these features for the prognosis of endogenous cancer?

Response: Thank you for your reminder. We further compared the prediction efficiency of the model we built with the 10 TMB related features. The results showed that compared with the single factor, our model had higher AUC(Area under the Curve) and higher prediction efficiency through the ROC (receiver operating characteristic curve) curve (Figure S1).

3.Are there any clinical interventions for these 10 TMB related characteristics?

Response: We thank the reviewer for their careful review, L1 cell adhesion molecule (L1CAM) is one of the first neural adhesion molecules described with essential functions in the development of the nervous system, and L1CAM is expressed in many human cancers and is often associated with bad prognosis[3]. Antibody therapy experiments in xenotransplanted mice bearing human ovarian, pancreatic, or cholangiocarcinoma tumors were successfully performed. In these studies, L1CAM mAbs were given either alone or in combination with cytotoxic drugs. It turned out that L1CAM-antibodies could significantly retard tumor growth and extended the survival of tumor-bearing animals[4-7].

ACTL8 is a member of the CT antigens, and Cancer-testis (CT) antigens are potential targets for cancer immunotherapy because of their restricted expression in immune-privileged germ cells and various malignancies[8]. T cells and antibodies against CT proteins are detectable in cancer patients, suggesting that the abnormal expression of CT antigens in tumors could induce adaptive immune response[9-11].

RYR1(Ryanodine Receptor 1) encodes a ryanodine receptor found in skeletal muscle. The encoded protein functions as a calcium release channel in the sarcoplasmic reticulum but also serves to connect the sarcoplasmic reticulum and transverse tubule. Accumulating evidence has demonstrated that intracellular Ca2+ homeostasis is altered in cancer cells, and the alteration is involved in tumor initiation, angiogenesis, progression, and metastasis[12], and RYR1is associated with Cancer-associated muscle weakness[13] and cancer cell apoptosis[14]. Treatment with 4-chloro-m-cresol(RyR agonist) can inhibit the growth of both breast cancer cells and prostate cancer in a dose-dependent manne[15].

KRT71, Keratins are intermediate filament proteins responsible for the structural integrity of epithelial cells and are subdivided into epithelial keratins and hair keratins. Keratins have long and extensively been used as immunohistochemical markers in diagnostic tumor pathology[16], and keratins have also been recognized as prognostic indicators in a variety of epithelial malignancies[17]. Although studies have shown that keratin may play a role in tumor chemotherapy resistance[18, 19], currently, there are no drugs targeting keratin in tumor therapy.

CLDN6, The claudin (CLDN) family, the backbone of tight junctions, consists of more than 20 members in humans and exhibits distinct expression patterns in tissue- and cell-type-specific manners. CLDN6 expression has been reported to be elevated in a variety of tumors, including gastric cancer[20], hepatocellular carcinoma[21], and knockdown of CLDN6 can significantly promote tumor proliferation. Therefore, CLDN6 may be a potential target for cancer therapy.

PNMA3(PNMA Family Member 3) belongs to the paraneoplastic antigen MA (PNMA) family, which shares homology with retroviral Gag proteins. Some members of the PNMA family members have been reported to play a role in cancer and apoptotic signaling[22]. Although sharing a relatively conserved amino acid sequence, some members of the PNMA family exhibit functional divergence, including members that are agonist or antagonist of apoptosis signaling when expressed in the cancer cell. Therefore, PMNA3 may act as a tumor apoptotic regulator to play its role in tumor inhibition, and it can be used as a candidate therapeutic target.

SLC22A16(Solute Carrier Family 22 Member 16) encodes a member of the organic zwitterion transporter protein family, which transports carnitine[23]. Unlike the restricted expression in healthy tissue, SLC22A16 is largely expressed in several cancer types, also originating from tissues that normally do not express this protein[24-27]. This denotes a profound rewiring of cancer cells concerning the carnitine linked metabolism. Reducing the expression of SLC22A16 can also significantly inhibit the growth of tumor cells, indicating that SLC22A16

may be a good target for cancer treatment[28].

LY6H(Lymphocyte Antigen 6 Family Member H), the Ly6H RNA is expressed at high levels compared to adjacent normal tissues in a multitude of tumors including ovarian, colorectal, gastric, breast, lung, bladder, brain and CNS, cervical, esophageal, head and neck, and pancreatic cancer[29]. The increased expression of LY6H was associated with poor survival in renal clear cell carcinoma and pancreatic ductal adenocarcinoma [30]. However, there are no drugs targeting LY6H yet, and further exploration may be needed in the future.

PDCL2(Phosducin Like 2) encodes a member of the phosducin-like protein family and is a putative modulator of heterotrimeric G proteins. At present, PDCL2 has rarely been studied in cancers. It has been reported that PDCL3 can be induced to express and regulate VEGFR-2 expression and promote angiogenesis under hypoxic conditions[31]. Therefore, further studies on PDCL2 are needed.

HIF3A(Hypoxia Inducible Factor 3 Subunit Alpha) belongs to the transcription factor family of hypoxia-inducible factors (HIFs), which regulate the cellular response to hypoxia[32]. Unlike HIF1, which has been studied extensively, therapeutic drugs and inhibitors against HIF3A have not been reported, which requires further study.

4.In addition to TMB, what other factors affect the prognosis of EC? Are there corresponding clinical interventions?

Response: Endometrial cancer, as one of the most common tumors in women, tends to occur in elderly women, and its prognosis is often related to the FIGO stage, clinicopathological type, age, estrogen exposure, obesity[33, 34], etc. The stage is the most important prognostic factor[35]. In 2013, the Cancer Genome Atlas (TCGA) published a seminal molecular study of endometrial carcinomas of endometrioid, serous, and mixed types. This revealed that endometrial carcinoma consists of four intrinsic molecular subtypes: POLE (ultramutated), microsatellite instability (hypermutated), copy-number low (also referred to as microsatellite stable or no specific molecular profile), and copy-number high (serous-like). These four molecular subtypes are of prognostic significance, with POLE tumors having the best and copy-number high, the worst prognosis[36].

The standard management of endometrial carcinoma is total hysterectomy and bilateral salpingo-oophorectomy with or without pelvic and para-aortic lymph node dissection. Those with more advanced disease may require chemotherapy or radiation therapy[35]. Increasingly, endometrial cancer is being diagnosed in younger women in whom preserving fertility may be an important consideration when deciding optimal management. Conservative management of endometrial carcinoma may be a therapeutic option in carefully selected women with well-differentiated endometrial cancer in the absence of any myometrial invasion or adnexal disease seen on imaging[37]. Bariatric surgery reduces the risk of endometrial cancer by up to 81% in obese women who attain and maintain a normal weight[38, 39]. Combined oral contraceptives provide durable protection against endometrial cancer for 30 years or more[40]. The first estrogen-based non-progestin HRT for non-hysterectomies women that contains estradiol and bazedoxifene has an effective protective effect on endometrium[41].

5.**Does TMB predict the prognosis of other gynecologic tumors except EC?**

Response: Thank you very much for your question. As a common tumor immunotherapy prognostic factor, TMB is also prognostic related to other gynecological tumors. In ovarian cancer, a high TMB was associated with a better clinical outcome of patients(p=0.007)[42]. Cervical cancer has a relatively high number of somatic mutations, being ranked sixth behind melanoma, lung, bladder, oesophageal and colorectal cancers[43], and a clinical trial that explored the association of high tissue TMB (tTMB-high) with outcomes in ten tumor-type-specific cohorts was performed, indicating that Cervical cancer patients with high TMB had better clinical remission rates after immune checkpoint blockade treatment[44].

6. Data of 529 EC patients were obtained from the TCGA database. How to select 529 patients and how to avoid selective bias?

Response: Thank you very much for your constructive opinions about the selection bias. It is a very worth considering problem, but it is very difficult for us to make accurate inclusion and exclusion criteria, so we chose all the samples with DNA mutation data in TCGA UCEC.

1. Ritterhouse LL: **Tumor mutational burden**. *Cancer Cytopathol* 2019, **127**(12):735-736.
2. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS *et al*: **Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer**. *Science* 2015, **348**(6230):124-128.
3. Altevogt P, Doberstein K, Fogel M: **L1CAM in human cancer**. *Int J Cancer* 2016, **138**(7):1565-1576.
4. Wolterink S, Moldenhauer G, Fogel M, Kiefel H, Pfeifer M, Lüttgau S, Gouveia R, Costa J, Endell J, Moebius U *et al*: **Therapeutic antibodies to human L1CAM: functional characterization and application in a mouse model for ovarian carcinoma**. *Cancer Res* 2010, **70**(6):2504-2515.
5. Arlt MJ, Novak-Hofer I, Gast D, Gschwend V, Moldenhauer G, Grünberg J, Honer M, Schubiger PA, Altevogt P, Krüger A: **Efficient inhibition of intra-peritoneal tumor growth and dissemination of human ovarian carcinoma cells in nude mice by anti-L1-cell adhesion molecule monoclonal antibody treatment**. *Cancer Res* 2006, **66**(2):936-943.
6. Knogler K, Grünberg J, Zimmermann K, Cohrs S, Honer M, Ametamey S, Altevogt P, Fogel M, Schubiger PA, Novak-Hofer I: **Copper-67 radioimmunotherapy and growth inhibition by anti-L1-cell adhesion molecule monoclonal antibodies in a therapy model of ovarian cancer metastasis**. *Clin Cancer Res* 2007, **13**(2 Pt 1):603-611.
7. Fischer E, Grünberg J, Cohrs S, Hohn A, Waldner-Knogler K, Jeger S, Zimmermann K, Novak-Hofer I, Schibli R: **L1-CAM-targeted antibody therapy and (177)Lu-radioimmunotherapy of disseminated ovarian cancer**. *Int J Cancer* 2012, **130**(11):2715-2721.
8. Yao J, Caballero OL, Yung WK, Weinstein JN, Riggins GJ, Strausberg RL, Zhao Q: **Tumor subtype-specific cancer-testis antigens as potential biomarkers and immunotherapeutic targets for cancers**. *Cancer Immunol Res* 2014, **2**(4):371-379.

9. Jäger D, Jäger E, Knuth A: **Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer**. *J Clin Pathol* 2001, **54**(9):669-674.
10. Mischo A, Kubuschok B, Ertan K, Preuss KD, Romeike B, Regitz E, Schormann C, de Bruijn D, Wadle A, Neumann F *et al*: **Prospective study on the expression of cancer testis genes and antibody responses in 100 consecutive patients with primary breast cancer**. *Int J Cancer* 2006, **118**(3):696-703.
11. Groeper C, Gambazzi F, Zajac P, Bubendorf L, Adamina M, Rosenthal R, Zerkowski HR, Heberer M, Spagnoli GC: **Cancer/testis antigen expression and specific cytotoxic T lymphocyte responses in non small cell lung cancer**. *Int J Cancer* 2007, **120**(2):337-343.
12. Cui C, Merritt R, Fu L, Pan Z: **Targeting calcium signaling in cancer therapy**. *Acta Pharm Sin B* 2017, **7**(1):3-17.
13. Waning DL, Mohammad KS, Reiken S, Xie W, Andersson DC, John S, Chiechi A, Wright LE, Umanskaya A, Niewolna M *et al*: **Excess TGF-β mediates muscle weakness associated with bone metastases in mice**. *Nat Med* 2015, **21**(11):1262-1271.
14. Shin DH, Leem DG, Shin JS, Kim JI, Kim KT, Choi SY, Lee MH, Choi JH, Lee KT: **Compound K induced apoptosis via endoplasmic reticulum Ca(2+) release through ryanodine receptor in human lung cancer cells**. *J Ginseng Res* 2018, **42**(2):165-174.
15. Abdul M, Ramlal S, Hoosein N: **Ryanodine receptor expression correlates with tumor grade in breast cancer**. *Pathol Oncol Res* 2008, **14**(2):157-160.
16. Moll R, Divo M, Langbein L: **The human keratins: biology and pathology**. *Histochem Cell Biol* 2008, **129**(6):705-733.
17. Karantza V: **Keratins in health and cancer: more than mere epithelial cell markers**. *Oncogene* 2011, **30**(2):127-138.
18. Cress AE, Roberts RA, Bowden GT, Dalton WS: **Modification of keratin by the chemotherapeutic drug mitoxantrone**. *Biochem Pharmacol* 1988, **37**(15):3043-3046.
19. Liu F, Fan D, Qi J, Zhu H, Zhou Y, Yang C, Zhu Z, Xiong D: **Co-expression of cytokeratin 8 and breast cancer resistant protein indicates a multifactorial drug-resistant phenotype in human breast cancer cell line**. *Life Sci* 2008, **83**(13-14):496-501.
20. Yu S, Zhang Y, Li Q, Zhang Z, Zhao G, Xu J: **CLDN6 promotes tumor progression through the YAP1-snail1 axis in gastric cancer**. *Cell Death Dis* 2019, **10**(12):949.
21. Huang L, Zhao C, Sun K, Yang D, Yan L, Luo D, He J, Hu X, Wang R, Shen X *et al*: **Downregulation of CLDN6 inhibits cell proliferation, migration, and invasion via regulating EGFR/AKT/mTOR signalling pathway in hepatocellular carcinoma**. *Cell Biochem Funct* 2020, **38**(5):541-548.
22. Pang SW, Lahiri C, Poh CL, Tan KO: **PNMA family: Protein interaction network and cell signalling pathways implicated in cancer and apoptosis**. *Cell Signal* 2018, **45**:54-62.
23. Console L, Scalise M, Mazza T, Pochini L, Galluccio M, Giangregorio N, Tonazzi A, Indiveri C: **Carnitine Traffic in Cells. Link With Cancer**. *Front Cell Dev Biol* 2020, **8**:583850.
24. Lal S, Wong ZW, Jada SR, Xiang X, Chen Shu X, Ang PC, Figg WD, Lee EJ, Chowbay B: **Novel SLC22A16 polymorphisms and influence on doxorubicin pharmacokinetics in Asian breast cancer patients**. *Pharmacogenomics* 2007, **8**(6):567-575.
25. Bray J, Sludden J, Griffin MJ, Cole M, Verrill M, Jamieson D, Boddy AV: **Influence of**

pharmacogenetics on response and toxicity in breast cancer patients treated with doxorubicin and cyclophosphamide. *Br J Cancer* 2010, **102**(6):1003-1009.

26. Sagwal SK, Pasqual-Melo G, Bodnar Y, Gandhirajan RK, Bekeschus S: **Combination of chemotherapy and physical plasma elicits melanoma cell death via upregulation of SLC22A16**. *Cell Death Dis* 2018, **9**(12):1179.
27. Zhang JZ, Wu ZH, Cheng Q: **Screening and identification of key biomarkers in nasopharyngeal carcinoma: Evidence from bioinformatic analysis**. *Medicine (Baltimore)* 2019, **98**(48):e17997.
28. Wu Y, Hurren R, MacLean N, Gronda M, Jitkova Y, Sukhai MA, Minden MD, Schimmer AD: **Carnitine transporter CT2 (SLC22A16) is over-expressed in acute myeloid leukemia (AML) and target knockdown reduces growth and viability of AML cells**. *Apoptosis* 2015, **20**(8):1099-1108.
29. Upadhyay G: **Emerging Role of Lymphocyte Antigen-6 Family of Genes in Cancer and Immune Cells**. *Front Immunol* 2019, **10**:819.
30. Luo L, McGarvey P, Madhavan S, Kumar R, Gusev Y, Upadhyay G: **Distinct lymphocyte antigens 6 (Ly6) family members Ly6D, Ly6E, Ly6K and Ly6H drive tumorigenesis and clinical outcome**. *Oncotarget* 2016, **7**(10):11165-11193.
31. Srinivasan S, Chitalia V, Meyer RD, Hartsough E, Mehta M, Harrold I, Anderson N, Feng H, Smith LE, Jiang Y *et al*: **Hypoxia-induced expression of phosducin-like 3 regulates expression of VEGFR-2 and promotes angiogenesis**. *Angiogenesis* 2015, **18**(4):449-462.
32. Uhlen M, Zhang C, Lee S, Sjöstedt E, Fagerberg L, Bidkhori G, Benfeitas R, Arif M, Liu Z, Edfors F *et al*: **A pathology atlas of the human cancer transcriptome**. *Science* 2017, **357**(6352).
33. Anderson B, Connor JP, Andrews JI, Davis CS, Buller RE, Sorosky JI, Benda JA: **Obesity and prognosis in endometrial cancer**. *Am J Obstet Gynecol* 1996, **174**(4):1171-1178; discussion 1178-1179.
34. MacKintosh ML, Crosbie EJ: **Prevention Strategies in Endometrial Carcinoma**. *Curr Oncol Rep* 2018, **20**(12):101.
35. Sorosky JI: **Endometrial cancer**. *Obstet Gynecol* 2012, **120**(2 Pt 1):383-397.
36. Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, Shen H, Robertson AG, Pashtan I, Shen R, Benz CC *et al*: **Integrated genomic characterization of endometrial carcinoma**. *Nature* 2013, **497**(7447):67-73.
37. Arora V, Quinn MA: **Endometrial cancer**. *Best Pract Res Clin Obstet Gynaecol* 2012, **26**(3):311-324.
38. Ward KK, Roncancio AM, Shah NR, Davis MA, Saenz CC, McHale MT, Plaxe SC: **Bariatric surgery decreases the risk of uterine malignancy**. *Gynecol Oncol* 2014, **133**(1):63-66.
39. Arthur R, Kirsh VA, Kreiger N, Rohan T: **A healthy lifestyle index and its association with risk of breast, endometrial, and ovarian cancer among Canadian women**. *Cancer Causes Control* 2018, **29**(6):485-493.
40. Iversen L, Sivasubramaniam S, Lee AJ, Fielding S, Hannaford PC: **Lifetime cancer risk and combined oral contraceptives: the Royal College of General Practitioners' Oral Contraception Study**. *Am J Obstet Gynecol* 2017, **216**(6):580.e581-580.e589.
41. Parish SJ, Gillespie JA: **The evolving role of oral hormonal therapies and review of**

conjugated estrogens/bazedoxifene for the management of menopausal symptoms. *Postgrad Med* 2017, **129**(3):340-351.

42. Bi F, Chen Y, Yang Q: **Significance of tumor mutation burden combined with immune infiltrates in the progression and prognosis of ovarian cancer**. *Cancer Cell Int* 2020, **20**:373.
43. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL *et al*: **Signatures of mutational processes in human cancer**. *Nature* 2013, **500**(7463):415-421.
44. Marabelle A, Fakih M, Lopez J, Shah M, Shapira-Frommer R, Nakagawa K, Chung HC, Kindler HL, Lopez-Martin JA, Miller WH, Jr. *et al*: **Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study**. *Lancet Oncol* 2020, **21**(10):1353-1365.