Impact of Age on Tumor Mutation Burden in Gastric patients:a secondary analysis based on a cross-sectional study

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Abstract

Background Gastric carcinoma is aggressive cancer with a poor prognosis. Recent years, immunotherapy has been validated effective in a proportion of patients. Tumor mutational burden (TMB), microsatellite instability (MSI) status and programmed cell death-ligand 1 (PD-L1) expression are potential biomarkers to predict efficacy to immunotherapy inhibitors (ICIs). This study was undertaken to explore the relationship between these biomarkers using the published data in a large patient cohort of gastrointestinal cancers. **Method** In total, 367 gastric cancer patients were examined in this study. MSI and TMB were assessed by next-generation sequencing (NGS). PD-L1 expression was determined by immunohistochemistry. **Results** The population was divided into four groups according to age, TMB-High and MSI-high participants were more frequent in older groups. After adjusting sex, differentiation, histology, specimen site, MSI status and PD-L1 expression, a non-linear relationship between age and TMB was found, which had an inflection point of 73 years. The effect sizes and the confidence intervals on the left and right sides of the inflection point were 0.06 (-0.01 to 0.13) and 0.56 (0.33 to 0.79), respectively. In different subgroups analysis, similar trends were observed. **Conclusion** The relationship between age and TMB is non-linear. Age was positively correlated with TMB when age was more than 73 years.

Background

In recent years, great progress has been achieved in optimizing cancer management by targeting immune checkpoint to release the immune system's brakes and restore the body's own anti-tumor immune responses in cancer treatment[1]. Several programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway inhibitors, such as nivolumab (anti-PD1), pembrolizumab (anti-PD1), atezolizumab (anti-PD-L1), avelumab (anti-PD-L1) and durvalumab (anti-PD-L1), have exhibited durable anti-tumor response and improved overall survival in a variety of malignancies, including gastric cancer[2-6].

Despite a steady decline, gastric cancer (gastric cancer) remains the third leading cause of cancer mortality in the world and a serious health burden in the world[7]. In the last few years, the immune checkpoint inhibitors (ICIs) have gained impetus in the treatment of advanced gastric cancer, and PD-L1 expression is considered as a potential predictive biomarker for efficacy to anti-PD-1 therapy[8]. However, more and more studies showed that PD-L1 expression does not fully explain the overall survival benefit gained from these ICIs. In order to maximize the clinical response for patients, more predictive and prognostic

biomarkers should be explored, such as tumor mutation burden (TMB), microsatellite instability (MSI) and Epstein-Barr virus (EBV) status [9-12].

In a large patient cohort of gastrointestinal cancers, TMB, MSI and PD-L1 expression were quantified and determined their interrelationship in gastrointestinal cancers. In their paper, the authors found that, MSI is the primary driver for TMB-high, and TMB-high rate varied widely among gastrointestinal carcinomas [13]. In this paper, we performed a secondary data analysis based on aforementioned data using the subset 'gastric cancer' patients.

Methods

Data source

Data were obtained from the DATADRYAD database (www.DataDryad.org), a website permitting users to download the raw data unlimitedly. Authors of the original research have waived copyright and ownership of the data. The Dryad data package Data (Dryad data package: Salem ME, Puccini A, Grothey A, Raghavan D, Goldberg RM, Xiu J, Korn WM, Weinberg BA, Hwang JJ, Shields AF, Marshall JL, Philip PA, Lenz H, 2018) was downloaded from: Landscape of tumor mutation load, mismatch repair deficiency, and PD-L1 expression in a large patient cohort of gastrointestinal cancers. <https://datadryad.org/resource/doi:10.5061/dryad.qt3v0t4>). Variables included in the database file were listed as follows: patient ID, primary tumorsite, specimensite, differentiation, histology, gender, age, TMB, MMR (MMR-stable or MMR-high) and PD-L1 expression (negative or positive).

Study population

This was a cross-sectional study involving 4123 patients conducted by Salem *et al* from 14 different gastrointestinal cancer sites in USA between 2009 and July of 2017. Tumors tested were consecutive samples submitted for molecular research. Pathologic and clinical diagnoses were acquired from corresponding pathology reports confirmed by board-certified path

ologists. In this paper, the subset 'gastric cancer' involving 367 patients was examined to explore the prevalence of MSI, TMB, and PD-L1 expression.

Measurement of TMB, MSI and PD-L1 expression

TMB, as measured by whole exon sequencing and counting all nonsynonymous missense mutations per tumor sample which haven't not been previously described as germline alterations, is associated with clinical benefit from immunotherapy. An Agilent SureSelect XT assay (Agilent, Santa Clara, CA, 592 genes and 1.4 MB sequenced/tumor) was used to calculate TMB. The threshold to define TMB-high was greater than or equal to 17 mutations/MB and was established by comparing TMB with MSI by fragment analysis in colorectal cancer cases. MSI was detected using over 7,000 target microsatellite loci and comparing with the reference genome hg19 from the University of California, Santa Cruz (Santa Cruz, CA) Genome Browser database. The number of microsatellite loci resulting from somatic insertion or deletion was counted for each tumor sample. Only insertions or deletions which would increase or decrease the number of repeats were considered to be of significance. Genomic variants in the microsatellite loci were detected using the same method in mutation detection. MSI results were compared with results from over 2,000 matching clinical cases analyzed with traditional PCR-based methods. The threshold to determine MSI was set to be ≥ 46 loci with insertions or deletions to generate a sensitivity of $>95\%$ and specificity of $>99\%$. Automated staining techniques with SP142 (Spring Biosciences) as primary antibody was used to assess PD-L1 expression. The staining intensity on the membrane of the tumor cell was scored according to a semiquantitative scale of 0–3 (0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining) and the percentage of positively stained cells was 5% [13].

Statistical analysis

Continuous variables were expressed as median (inter-quartile range), and categorical variables were expressed in frequency or as a percentage. Kruskal Wallis H test and chi-square tests were applied to detect the statistical differences between different groups. Generalized additive model (GAM) was used to identify the non-linear relationship between age and T

MB. If the non-linear correlation was found, a two-piecewise linear regression model was used to examine the threshold effect of the age on TMB in terms of the smoothing plot. The subgroup analyses were performed using the calculated threshold, and interaction between subgroup was also inspected. All of the analyses were performed with the statistical software packages R (<http://www.R-project.org>, The R Foundation) and EmpowerStats (<http://www.empowerstats.com>, X&Y Solutions, Inc., Boston, MA). P values less than 0.05 (two-sided) were considered to be statistically significant.

Results

Tumor characteristics

In total, this cohort was composed of 143(38.96%) female patients and 224(61.04%) male patients. The median age was 62 years(IQR,51-73). Most specimens obtained from primary tumor sites (64.85%) comparing with the metastatic sites (35.15%). 330 samples were classified to adenocarcinoma, and the remaining 37 cases included 1 mixed adenocarcinoma/large cell neuroendocrine carcinoma, 4 neuroendocrine carcinoma and 6 adenosquamous carcinoma, the other 26 cases can't be divided into any group of the above. In the cohort, 155 tumor samples were recorded as "poorly differentiated", 29 cases "moderately differentiated", 24 cases "moderately to poorly differentiated", 1 case "well to moderately differentiated", the remaining 158 cases without specific records.

About the TMB examination, as 17 mutations/MB the cutoff point, TMB-High group comprised 8.45% of the cohort. About the MSI and PD-L1 results, 29 cases were classified as MSI-High and 34 cases were classified as positive group (Table 1).

Table 1 Characteristics of patients

Characteristic	No. (%)
No. of patients	367
Age(IQR)	62(51-72)
Gender	
female	143(38.96)
male	224(61.04)
Specimensite	
primary	238(64.85)
metastatic	129(35.15)
Differentiation	
poor	155(42.23)
moderate	29(7.90)
moderate-poor	24(6.54)
well-moderate	1(0.27)
unclear	158(7.08)
Histology	
adenocarcinoma	330(89.92)
adenosquamous carcinoma	6(1.63)
neuroendocrine carcinoma	4(1.09)
mixed adenocarcinoma/large cell neuroendocrine carcinoma	1(0.27)
unclear	26(10.08)
TMB(IQR)	7(6-9)
TMB-High	31(8.45)
TMB-Low	336(91.55)
MSI	
stable	336(91.55)
high	29(7.90)
equivocal	2(0.54)
PD-L1 expression	
negative	319(86.92)
positive	34(9.26)
unclear	14(3.81)

Noted:IQR,inter-quartile range.

Interrelationship between TMB, MSI, and PD-L1 was displayed in Figure 1. It was shown that the distribution of TMB-high group was highly consistent with that of MSI-high, but not with PD-L1 positive cases. Therefore, the integration of TMB or MSI would broaden the potentially beneficial population who might response to ICIs.

The results of relationship between age and TMB

Firstly, the population was divided into four groups with the age increasing. TMB-high and MSI-high were more frequent in older groups. Because TMB was continuous variable, we analysed the non-linear relationship between age and TMB. We found that the relationship between age and TMB was non-linear (after adjusting sex, differentiation, histology, specimensite, MSI-status, PD-L1 expression). By using a two-piecewise linear regression model, we calculated that the inflection point was 73 years. On the left of the inflection point, the effect size, 95%CI and P value were 0.06, -0.01 to 0.13 and 0.0731, respectively. However, we observed a significantly positive relationship between age and TMB on the right side of the inflection point (0.56, 0.33 to 0.79, <0.001) (Table 2 and Fig 2).

Table 2 Characteristics of variables in different age groups

Age (quartile)	Q1	Q2	Q3	Q4	P*
N	88	95	92	92	
Age[IQR]	43.00 (37.00-47.00)	57.00 (54.00-59.50)	67.00 (65.00-69.25)	77.50 (75.00-81.00)	<0.001
Sex					0.008
female	48 (54.55%)	32 (33.68%)	32 (34.78%)	31 (33.70%)	
male	40 (45.45%)	63 (66.32%)	60 (65.22%)	61 (66.30%)	
Differentiation					0.572
Others ^a	46 (52.27%)	57 (60.00%)	57 (61.96%)	52 (56.52%)	
poor	42 (47.73%)	38 (40.00%)	35 (38.04%)	40 (43.48%)	
Histology					0.062
others ^b	12 (13.64%)	13 (13.68%)	3 (3.26%)	9 (9.78%)	
adenocarcinoma	76 (86.36%)	82 (86.32%)	89 (96.74%)	83 (90.22%)	
Specimensite					0.132
primary	48 (54.55%)	63 (66.32%)	63 (68.48%)	64 (69.57%)	
metastatic	40 (45.45%)	32 (33.68%)	29 (31.52%)	28 (30.43%)	
TMB	6.00	7.00	7.00	9.00 (6.00-12.00)	<0.001
low	(5.00- 8.25)	(6.00- 9.00)	(5.75-9.00)		
high	86 (97.73%)	91 (95.79%)	83 (90.22%)	76 (82.61%)	
MSI	2 (2.27%)	4 (4.21%)	9 (9.78%)	16 (17.39%)	<0.001
stable	87 (98.86%)	92 (96.84%)	84 (91.30%)	75 (81.52%)	
high	1 (1.14%)	3 (3.16%)	8 (8.70%)	17 (18.48%)	
PD-L1 expression					0.897
negative	79 (89.77%)	82 (86.32%)	80 (86.96%)	78 (84.78%)	
positive	6 (6.82%)	8 (8.42%)	9 (9.78%)	11 (11.96%)	

Noted:IQR,inter-quartile range; P* was calculated by Kruskal-Wallis H test;others^ainclude moderate,moderate-poor,well-moderate and unclear differentiated cases;others^b,include adenocarcinoma,neuroendocrine carcinoma,mixed adenocarcinoma/large cell neuroendocrine carcinoma and unclear pathology types;TMB,

tumor mutation burden; MSI,microsatellite instability; PD-L1,programmed cell death ligand.

Table 3 The results of two-piecewise linear mode

Inflection point of age (Per 1 year change)	Effect size (β)	95%CI	P value
<73	0.06	-0.01, 0.13	0.0731
≥ 73	0.56	0.33, 0.79	<0.0001

CI: Confidence interval;we adjusted sex, differentiation,histology,specimensite, MSI-status,PD-L1 expression

As is shown in Table 4, the test for interactions was not statistically significant for sex, differentiation,histology,PD-L1 expression (P for interaction =0.70, 0.25, 0.64 and 0.22,respectively). The consistent result was observed in different subgroups divided by gender,PD-L1 expression and differentiation.

Table 4 Results of subgroup analysis and interaction analysis

	No of participants	Effect size(95%CI)	P for interaction
Sex			
female	143	3.34 (1.13, 9.86)	0.6950
male	224	4.53 (1.54, 13.33)	
PD-L1 expression			
negative	319	2.49 (1.01, 6.15)	0.2239
positive	34	8.00 (1.47, 43.68)	
Differentiation			
poor	155	5.05 (1.93, 13.17)	0.2489
others ^a	212	2.33 (0.63, 8.60)	
Histology			
adenocarcinoma	330	4.29 (1.89, 9.72)	0.6369
others ^b	37	2.89 (0.39, 21.29)	

CI: Confidence interval;other^ainclude moderate,moderate-poor,well-moderate and unclear differentiation;others^b,include adenosquamous carcinoma,neuroendocrine carcinoma,mixed adenocarcinoma/large cell neuroendocrine carcinoma and unclear pathology types;PD-L1,programmed cell death ligand

Discussion

In recent years, cancer immunotherapy has revolutionized the oncology landscape by rebalancing the host immune system. Blocking immune checkpoints such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and PD-1/PD-L1, has proven effective in a series of solid malignance[16]. Recent studies suggested that ICIs might become a future therapeutic option in gastric cancer. Based on results from KEYNOTE-059 trial, Food Drug Administration (FDA) approved pembrolizumab after two or more prior lines of therapy for patients whose tumor overexpressed PD-L1 in advanced gastric and gastroesophageal junction cancer[17]. However, the relationship between PD-L1 immunohistochemical positivity and response to ICIs have been shown almost invariably in patients with either metastatic melanoma or Non-small Cell Lung Cancer(NSCLC)[18]. Because gastric cancer also belongs to epithelial malignancies with many similarities in etiology, morphology and mutational profile with NSCLC, it is rational to extrapolate the correlations between PD-L1 overexpression and ICIs response. In KEYNOTE 059 and Checkmate-032 studies, the objective remission rate(ORR) and Disease control rate(DCR) were respectively higher in PD-L1+ tumors, however, in KEYNOTE-061 and JAVELIN GASTRIC 300 studies, no clinical improvement was observed in PD-L1+ tumors[17, 19]. In all, the accessible data suggest that PD-L1 may not be the perfect biomarker for selecting patients in gastroesophageal cancer patients for immunotherapy. Therefore, more studies predicting immunotherapy efficacy are going ahead, such as MSI-status, EBV positivity, tumor infiltrating lymphocytes (TILs), TMB and so on[20-22]. Taking any of these indicators into consideration would hypothetically maximize the clinical response of a specific subgroup of patients.

In Salem *et al* study, TMB, MSI-status and PD-L1 expression were examined, as in Figure 1, the distribution of MSI-status was obviously more consistent with TMB than PD-L1 expression[13]. Even though MSI-status was tightly associated with TMB, it was possible to observe TMB-high subjects in MSS tumors[23]. Therefore, the combination of TMB, MSI-status and PD-L1 expression would broaden more potential subjects who might benefit from immunotherapy.

Findings in selected cancer types suggest that TMB may predict clinical response to ICI[23]. Higher levels of mutations were believed to induce the expression of immunogenic and c

ancer-specific neoantigens, leading to a robust antitumor immune response. In the first edition of the latest National Comprehensive Cancer Network(NCCN) guidelines for NSCLC in 2019, Tumor Mutation Burden (TMB), as Emerging Biomarker, was first included in the guidelines for screening suitable patients who could be treated with combination of Nivolumab combined with Ipilimumab. The TMB cutoff point associated with improved survival varied markedly between cancer types, for example, the cutoff point was chosen as 20 mutations/MB in NSCLC[24]. In this analysis, 17 mutations/MB was used as the cutoff point based on previous study.

In this secondary analysis, the impact of age on TMB in gastric cancer patients was first founded. As far as we know, there were limited studies about the impact of age on TMB. Similarly, Wang *et al* reported that younger gynecologic cancer patients (age <40 years) had a significantly lower TMB than older patients (age $\geq$ 40 years)[25]. In an initial clinical cohort of 102,292 samples for 167 distinct cancer types, a significant increase in TMB associated with increased age was documented[26]. This phenomenon could be explained by age-related somatic mutations in cancer genome. Studies in mice and fruit flies have shown that somatic mutations accumulate with age in an issue-specific style, with respect to both the rate of age-related increase and the types of mutations found to accumulate[27, 28]. The report from a 6,969 samples' study showed that the number of somatic mutations in tumors was distinctly higher when the tumor derived from an old patient, comparing with a young one[29, 30]. The molecular biological mechanisms of aging involving somatic mutation may include p16(Ink4a) mutation, dysfunction of P53, DNMT3A mutations, SIRT1 gene disorder and some unspecified mechanisms[31-34]. TMB calculation is conducted by counting all nonsynonymous missense somatic mutations. So we thought it is plausible that TMB could mirror the somatic mutation in genome. As far as we know, this is the first report to show the influence of age on TMB in gastrointestinal cancers. It gives us some hint that, immunotherapy should be given priority to older patient when anti-tumor decision making, comparing to young patients. But the correlation might vary to some extent between cancer types, for example, Puccini *et al* found an opposite result that higher rates of TMB-high tumors occur in younger

patients than in old patients[14].So there maybe some other unilluminated mechanism to conceal the age-related increase in mutation burden.

There are some limitations in this study. Firstly, due to the nature of cross-sectional study, we provide only weak evidence between age and TMB, and it is difficult to distinguish cause and effect. Secondly, the tumor sample collected is restricted to the American, so the generalizability is restricted. Lastly, this study is a secondary analysis based on published data,so variables not included in the raw datasheet cannot be analysed, such TNM stage, EBV status and Lauren type.

Conclusion

A non-linear relationship between age and TMB was found in this manuscript,which had a n inflection point of 73 years, after adjusting sex, differentiation,histology,specimensite, MSI-status and PD-L1expression.As far as we know, this is the first report demonstrating the correlation between age and TMB in smooth curve in various types of tumors. So we think that immunotherapy should be considered for patients when anti-tumor scheme making.

Abbreviations

TMB:tumor mutation burden; MSI:microsatellite instability; PD-1:programmed cell death 1;PD-L1:programmed cell death ligand; ICIs:immunocheckpoint inhibitors;

Declarations

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Availability of data and materials

The dataset supporting the conclusions of this article is available from: Landscape of tumor mutation load, mismatch repair deficiency, and PD-L1 expression in a large patient cohort of gastrointestinal cancers.<https://datadryad.org/resource/doi:10.5061/dryad.qt3v0t4>).

Authors' contributions

The study was conceived and designed by Baolan Li, Tongmei Zhang and Mingming Hu. Mingming Hu, analyzed the data and drafted the manuscript. Jie Li and Yuan Yang preliminary reviewed the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Venn diagram showing the overlap of PD-L1 expression, MSI-high, and TMB-high in gastric cancer, with all three markers tested. N indicates the number of cases within each category.

Figure 2

The relationship between age and TMB. A nonlinear relationship between them was detected after adjusting for sex, differentiation,histology,specimensite, MSI -status and PD-L1 expression.