Impact of Tumor Grade Distribution on Genetic Alterations in Clear Cell Renal Cell Carcinoma and Prostate Cancer

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Abstract. Background/Aim: A genomic analysis based on next-generation sequencing is important for deciding cancer treatment strategies. Cancer tissue sometimes displays intratumor heterogeneity and a pathologic specimen may contain more than two tumor grades. Although tumor grades are very important for the cancer prognosis, the impact of higher tumor grade distribution in a specimen used for a genomic analysis is unknown. Patients and Methods: We retrospectively analyzed the data of 61 clear cell carcinoma and 46 prostate cancer patients that were diagnosed between December 2018 and August 2022 using the GeneRead Human Comprehensive Cancer Panel or SureSelect PrePool custom Tier2. Genome annotation and curation were performed using the GenomeJack software. Results: Tumor mutation burden (TMB) was increased in proportion to the higher tumor grade distribution in grade 2 clear cell renal cell carcinoma (ccRCC). In PC, Grade Group 3/4 specimens that included an increased distribution of Gleason pattern 4 had more frequent gene mutations. Conclusion: Our results suggest the importance of selecting the maximum distribution of higher tumor grade areas to obtain results on the precise gene alterations for genomics-focused treatments.

Despite recent advances in cancer treatment, advanced cancers are still incurable. Gene mutations in cancer are promising treatment targets (1, 2); thus, the precise detection of genetic alterations gives cancer patients a greater chance to receive genomics-focused treatment. Next-generation sequencing (NGS) technologies have played an important role in the detection of the genetic alterations of cancer (3). Clinically used cancer panel tests based on NGS are performed starting with DNA extracted from formalin-fixed paraffin-embedded sections or with circulating cell-free DNA extracted from blood (1, 4). In samples with <20% tumor cells in formalin-fixed paraffin-embedded sections, the sensitivity in the detection of copy number alterations (CNAs) or mutations may decrease (5, 6). The targeting of the area of the section to be used as the specimen for DNA extraction is usually determined by pathologists in order to maximize tumor cell content. Meanwhile, tumor grade, which is mostly defined by the structure and cytological features including nuclear or morphological atypia, is one of
strongest predictive factors in many cancers. Although, pathologically high-grade cancer, which shows an aggressive nature with increased genomic instability and mutations (7-10), is likely to show more genetic alterations, the relationship of the area of high-grade tumor distribution in specimens and genetic alterations has not been studied.

In clear cell or papillary renal cell carcinoma (RCC), tumor grading based on nuclear atypia (grades 1-3), and with features of extreme nuclear pleomorphism, multinucleated giant cells, and/or rhabdoid and/or sarcomatoid differentiation (grade 4) is widely used in the World Health Organization/International Society of Urological Pathology (WHO/ISUP) grading system (11). In the grading system, the highest tumor grade is assigned when more than one of the grading features exists in a tumor. Mutational heterogeneity, with the exception of VHL, among different regional tumor grades of clear cell RCC (ccRCC) has been reported (12). On the other hand, Ball et al. reported no association between the Fuhrman grade—which is defined by morphological atypia (glandular architecture and microscopic appearance). The Gleason score system has been refined with the establishment of the PC grade group (GG) (11). Prostate cancer with Gleason score ≤6, 3+4, 4+3, 8, and 9-10 are categorized to GG 1, 2, 3, 4, and 5, respectively. Some genetic alterations, including mutations in DNA repair genes, are associated with advanced stage and higher Gleason grade in PC (8, 9, 14).

In this context, it seems natural to select samples with the area that shows the maximum distribution of the highest tumor grade because tumor heterogeneity is often observed in many cancers (15-17). Many studies using different samples from individual patients demonstrated that a high tumor grade was associated with increased genomic alterations; however, the impact of intra-tumor grade distribution in the pathological specimen on genetic alterations has not been elucidated. In this study we hypothesized that increased distribution of the higher tumor grade in urologic cancer specimens would affect the detection of genetic alterations.

**Patients and Methods**

**Patients and samples**

The study protocol was reviewed and approved by the Institutional Review Board of Central Japan International Medical Center (No.2022-44). Each patient had provided written informed consent. This retrospective study was conducted by reviewing the data of ccRCC and PC patients who underwent surgery or biopsy followed by a genome profiling test between December 2018 and August 2022. The tumor grades of ccRCC and PC were diagnosed based on the 2016 WHO/ISUP grading system. Patients pre-treated before surgery or biopsy were excluded from this analysis.

**DNA isolation and next-generation sequencing-based multiplex gene assay.** Prior to DNA extraction, pathologists investigated the tissue tumor cell content on hematoxylin and eosin-stained slides. At the time, the distribution of each tumor grade was not considered. The area that predominantly included tumor cells was then macro-dissected from 10-μm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections. DNA was extracted using Maxwell RSC DNA FFPE Kit-PKK, Custom (Promega, Fitchburg, WI, USA). A multiplex gene assay based on NGS was reported previously (18, 19). After quantification of purified DNA using an Agilent 4200 TapeStation, DNA libraries were prepared with DNA (DNA integrity number >3.1) for subsequent genomic sequencing. From December 2018 to December 2021, gene amplification was performed using a GeneRead Human Comprehensive Cancer Panel (160 genes; Qiagen, Hilden, Germany). From January 2022 to August 2022, it was performed using SureSelect PrePool custom Tier2 (143 genes; Agilent, Santa Clara, CA, USA). Targeted amplicon exome sequencing for cancer-related genes was performed using the Illumina Miseq sequencing platform (Illumina, San Diego, CA, USA). Genome annotation and curation was performed using GenomeJack (Mitsubishi Space Software, Tokyo, Japan) (20, 21). Based on this platform, high scoring (≥2.0) gene mutations were noted as actionable gene mutations. Since two different panels were used, the common target gene mutations in the panels were analyzed in this study.

**Statistical analysis.** All statistical analyses were performed using the Graph Pad Prism software program (version 7.03, Graph Pad Software, San Diego, CA, USA). Comparisons among the three groups were performed using the Kruskal–Wallis test with Dunn’s multiple comparison test. Categorical variables were analyzed using the chi-squared test. p-Values of <0.05 were considered to indicate statistical significance.

**Results**

**Patient cohort and samples.** Eighty-three cases of RCC and 419 cases of PC were diagnosed by either surgery or biopsy. Of these, 69 cases of RCC and 56 cases of PC were analyzed by cancer panel tests. After applying the exclusion criteria, data from 61 cases of clear cell RCC (ccRCC) and 46 cases of PC were included in this study (Figure 1). All specimens were pathologically reviewed by a pathologist (S Sugiyama) who evaluated the distribution of each tumor grade in the macro-dissected area in a blinded manner. Representative data of specimens of ccRCC and PC for the cancer panel tests are shown in Figure 2. Grade 2 ccRCC specimens
included a different ratio grade 2 dominant area (Figure 2A and B). Grade group 2/3 PC included Gleason pattern 3- and 4-dominant specimens (Figure 2C and D).

**TMB and CNA count in ccRCC.** The tumor mutation burden (TMB) in specimens of each ccRCC tumor grade was analyzed. The TMB in grade 3 or 4 ccRCC was increased in comparison to that in grade 1 or 2, but the difference was not statistically significant (Figure 3A). Since the WHO/ISUP grading system of RCC notes the highest tumor grade, grade 2 ccRCC tumors include grade 1 tumors with a small amount of grade 2, to 100% grade 2 tumors. To analyze the relationship of tumor grade distribution and TMB, we investigated the TMB only in the grade 2 group. The ratio of grade 2 distribution area was from 20–100% (the rest of the areas were grade 1). The median TMB in specimens with ≥50% grade 2 was significantly higher than that in those with <50% grade 2 (Figure 3B). In addition, a strong positive correlation between the TMB and the ratio of the grade 2 distribution area was observed (Figure 3C). We also investigated the CNA count using the same dataset. There was no significant correlation between the CNA count and the ratio of the grade 2 distribution area (Figure 3D-F).

**Gene mutations in ccRCC.** We analyzed the frequency of actionable gene mutations in each tumor grade. The number of gene mutations in WHO/ISUP grade 1, grade 2 and grade 3/4 were 22 in 37 (59.5%), 14 in 16 (87.5%) and 4 in 8 (50.0%), respectively (Figure 4A). The frequency of gene mutations in grade 2 tended to be increased in comparison to G1; however, the difference was not statistically significant. Mutations in BAP1, PBRM1, SETD2 and PTEN play important roles in tumor evolution in ccRCC in addition to VHL mutation, which is the most common gene mutation in this type of cancer (18, 22). VHL mutation was observed 54.1%, 68.8 and 37.5% of grade 1, grade 2 and grade 3/4, respectively. The rates of any mutation in the above 4 genes were 18.9%, 38.0 and 25.0% in grade 1, grade 2 and grade 3/4, respectively (Figure 4B). Subsequently, the mutation rate was analyzed according to the ratio of the grade 2 distribution area in grade 2. In 5 specimens with grade 2 ≥20%, 4 VHL mutations (80%) and a PBRM1 mutation (20%) were detected. On the other hand, 7 mutations in VHL (63.6%), 2 mutations in BAP1 and SETD2, and 1 mutation in PBRM1 and PTEN (45.4% in total) were detected in 11 specimens with ≥30% grade 2 distribution (Figure 4C).

**TMB and CNA count in PC.** The prostate cancer grade group (GG) is a refined version of the Gleason score system. Features of Gleason ≤6, 3+4, 4+3, 8 (3+5, 4+4, 5+3), and 9 (4+5, 5+4)-10 (5+5) are classified to GG1-5, respectively. The TMB and CNA count according to each GG are shown in Figure 5A and C, respectively. Although the TMB and CNA count were high in some GG4 or GG5 samples, the median values showed no significant difference. There was a weak but positive correlation between TMB and GG (Figure 5B and D). The Gleason score, which is the basis of the GG is defined by adding together the 2 most common grades.
Gleason patterns with >5% distribution; thus, the distribution of the second component may range from 5-45% in Gleason score 3+4 (GG2) or 4+3 (GG3). However, the distribution of the Gleason pattern in the specimen used for the cancer panel test, a part of whole tumor, is not noted. To study whether the range of the second Gleason pattern in the specimen affected the TMB or CNA count, we analyzed the correlation between TMB/CNA count and the ratio of Gleason pattern 4 distribution (Figure 5E and F).

Gene mutations in PC. We analyzed the frequency of actionable gene mutations in each GG of PC; the number of cases with mutations is shown in Figure 6A. No gene mutation was detected in GG1, while higher GGs showed more mutations. Specimens with ≥50% Gleason pattern 4 or with a Gleason pattern 5 component had significantly more mutations in comparison to specimens with <50% Gleason pattern 3 (Figure 6B). The frequency of cases with mutations in GG2/3 according to the ratio of Gleason pattern 4 is shown in Figure 6C. Specimens with ≥70% Gleason pattern 4 had significantly more mutations in comparison to those with <70% Gleason pattern 4 in GG2/3.

Discussion
For the best yield of tumor-derived DNA extraction, pathologists need to select high tumor content areas in specimens. A higher tumor grade reflects the essence of genetic alterations from the most critical features of the cancer, and the precise detection of genetic alterations is important for genomics-focused treatments. However, whether a higher tumor grade distribution in the specimen impacts the sensitivity of the detection of genetic alterations has not been elucidated. For genomic analyses, the results of
Figure 3. The tumor mutation burden (TMB) and copy number alteration (CNA) count in clear cell renal cell carcinoma (ccRCC). A: TMB in grade 1, grade 2 and grade 3/4. B: TMB in cases with 20-40% grade 2 distribution area and 50-100%. C: The correlation between TMB and the ratio of the G2 distribution area in G2 ccRCC. D: The CNA count in grade 1, grade 2 and grade 3/4. E: CNA count in cases with 20-40% grade 2 distribution area and 50-100%. F: The correlation between CNA count and the ratio of the G2 distribution area in G2 ccRCC.
our study indicate that pathologists need to consider the
distribution ratio of higher tumor grade areas when more
than two different morphological features are present in a
specimen, in addition to the tumor cell content.

In our cohort of patients with ccRCC, the TMB increased
with higher ratio of grade 2 distribution. The TMB is a known
favorable predictive factor of immune checkpoint inhibitor in
several cancers, but its relevance in RCC is controversial (23).
We demonstrated that the TMB may depend on the tumor
grade distribution; thus, the evaluation of TMB using
specimens with maximum distribution ratio of higher tumor
grade may provide different results regarding the role of TMB
in the prediction of ccRCC treatment. VHL degrades hypoxia-
inducible factor (HIF), which regulates the expression of a
large number of target genes related to tumor progression (24,
25). In line with a previous study, VHL was the most common
mutated gene in ccRCC and was found in 55.7% in our cases
(26). VHL mutation is important in the initial clonal expansion
following the loss of chromosome 3p, which includes the
VHL gene (22). Since the mutation occurs at the initial stage
of tumorigenesis, i.e., decades before diagnosis, the frequency
of VHL mutation was consistent through all tumor grades.
Belzutifan, a potent, selective small molecule inhibitor of HIF-
2α, has shown promising anti-cancer activity in ccRCC (27).
As long as only VHL is targeted, it may not be necessary to
select the higher tumor grade area for a genetic analysis in
ccRCC. On the other hand, clonal or subclonal drivers of
mutations of BAP1, PBRM1, PTEN, or SETD2 accelerate the
rapid progression of RCC. Mutations in these genes were
likely to be found in specimens with higher tumor grade
(grade 2) distribution in WHO/ISUP grade 2 ccRCC.
Treatments based on these mutations are being developing in
many cancer types (28-31). In this context, the higher-grade
tumor content is preferred to detect gene mutations other than
the VHL mutation, which would maximize the chances of
achieving genomics-focused treatments in RCC.

Figure 4. Gene mutations in clear cell renal cell carcinoma (ccRCC). A:
The numbers of cases with or without gene mutation in each tumor grade
do ccRCC. The statistical value was calculated using Fisher’s exact test
with the Bonferroni post hoc test. B: The frequency of cases with VHL
mutation or a mutation of BAP1, PBRM1, SETD2, or PTEN in each tumor
grade of ccRCC. C: The frequency of cases with VHL mutation or a
mutation of BAP1, PBRM1, SETD2, or PTEN in specimens with ≤20%
grade 2 distribution and ≥30% grade 2 distribution.
Figure 5. Tumor mutation burden (TMB) and copy number alteration (CNA) count of prostate cancer (PC). A: TMB in PC diagnosed as grade group 1-5. B: The correlation between TMB and grade group. C: The CNA count in PC diagnosed as grade group 1-5. D: The correlation between CNA count and grade group. E: The correlation between TMB and the ratio of Gleason pattern 4 distribution area in G3/4 PC. F: The correlation between CNA count and the ratio of Gleason pattern 4 distribution area in G3/4 PC.
Since not all tumor grades are diagnosed by nuclear atypia like ccRCC, we verified our results using a prostate cancer dataset, in which the grade is defined by structural morphology; the so-called Gleason grading system. We identified no significant differences between GGs and TMB or CNA count, whereas the frequency of mutations was higher in specimens with an increased Gleason pattern 4 distribution area in GG2/3. Higher GG, which denotes an increased number of gene mutations, might be suitable for targeted therapies. In GG3/4, Gleason pattern 3-dominant specimens (≥70% Gleason pattern 3) had only two mutations in ECT2L and KDM6A in 11 samples. In Gleason pattern 4-dominant specimens (≥70% Gleason pattern 4), mutations were detected in 8 out of 10 cases. The mutations were in ARID1A, BRAF, CDK12, GRIN2A, IDH1, KDM6A, SMARCA4, and SPOP. Some of them are targets of approved or developing novel targeted therapies (32). The SPOP mutation was also reported to be a marker of a superior response to androgen deprivation therapy plus androgen receptor axis-targeted therapies, which are essential treatments for PC (33). It is preferred to maximize the distribution area of Gleason pattern 4 in a specimen to detect more mutations by a cancer panel test in GG2/3 PC. Conversely, that type of sample collection may not be necessary for a TMB analysis in PC.

We also analyzed whether these results are compatible with other types of cancer in 44 untreated breast cancer specimens (luminal A: 6, luminal B: 12, luminal HER2: 4, HER2: 4, triple negative: 18). There was no association between genetic alterations (TMB, CNA count, and frequency of gene mutation) and the nuclear features/amount of gland formation, which are part of the components of the Nottingham Histologic Score system (data not shown). This result, which is inconsistent with ccRCC or PC data, could be explained by the following reason: our breast cancer dataset included mostly aggressive cancer types. Nevertheless, sample collection from the highest nuclear or structure grade area with the maximum distribution is worth consideration in cancer panel tests.
Study limitations. It was a retrospective study based on a small sample size. Tumor cell sampling was performed by macro-dissection, and the tumor fraction data on the individual tumor grades was absent. Lower tumor cell content may affect the results of cancer panel tests. In addition, mutation calling in our dataset may differ from the results obtained using other mutation calling algorithms. Therefore, further clinical studies with larger datasets of cancer panel tests that are widely used in the clinical setting are needed to validate our results, in which several of our findings show several borderline significance or associations.

Conclusion

Genomic medicine contributes to accurate cancer diagnosis, effective treatment, and cancer prevention. Intratumor heterogeneity of genomic alteration may have an impact on cancer genomic medicine. Our study’s findings suggest that maximizing higher-grade tumor distribution as well as tumor cell content in a specimen is important for genomic analysis of ccRCC and PC. Precise results based on prior knowledge and our findings may contribute to better genomics-focused pharmacology.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors’ Contributions

Conception and design: KMi. Data analysis and interpretation: KMi, SSu, SSa, IS, HF and KH. Data curation: IS, YK and HN. Clinical evaluations and treatment: KMi, Ssu, KK, SK, SY, AM, MT, KS, TY, HE, KMa, TY SI and TD. Article writing: KMi. All Authors read and approved the final manuscript.

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