Superiority of rat over murine model for studies on the evolution of cancer genome

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ABSTRACT
Evolution of the species and carcinogenesis are similar in that genomic alterations are the key events. Oxidative stress derived from various etiologies is one of the major causes of carcinogenesis by inducing mutations in the genome. Persistent oxidative stress in the renal proximal tubules through Fenton reaction catalysed by ferric nitritotriacetate (Fe-NTA) generates renal cell carcinoma (RCC) in mice and rats. Here, in order to observe the species difference in oxidative stress-induced carcinogenesis and to obtain an insight regarding the characteristics of each species, we compared the genomic alterations using array-based comparative genome hybridisation among RCCs in Mutyh knockout/wild-type mice (C57BL/6 background) induced by Fe-NTA, RCCs in F1 hybrids of Brown-Norway/Fischer-344 wild-type rats and clear cell renal cell carcinoma (CCRCC)/papillary renal cell carcinoma (PRCC) of humans. The average deviated fraction of genomic segments, either loss or gain, from the standard biallelic position was 0.220 (N = 4), 0.304 (N = 11), 0.283 (N = 12), and 0.261 (N = 5), respectively, for murine RCC, rat RCC, human CCRCC, and human PRCC. Notably, gain/loss ratio was remarkably different as indicated by 0.0820, 0.161, 0.821, and 4.44, respectively. These data suggest that higher species require more genomic alterations with amplification preference for renal carcinogenesis. Further studies are necessary to identify the molecular mechanisms whether the present results depend on cellular functional differences, etiology of carcinogenesis or the target cells in carcinogenesis.

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Introduction
Cancer has been one of the top causes of human mortality worldwide after the conquest of major infectious diseases, including tuberculosis (http://www.who.int/mediacentre/factsheets/fs297/en/). Cancer is the first place of mortality in Japan since 1981, and cancer mortality is still increasing according with the prolonged average lifetime (http://www.fpocr.or.jp/pdf/p21/cancer_statistics_2016.pdf). Cancer is now recognised as a disease of the genome whereas epigenetic changes are also important. We are proposing that one of the major mutational pressures on the genome when integrated during the whole life is caused by oxygen and iron that we always use [1]. Hemoglobin in red blood cells holds ~60% of iron of our body as heme to transport oxygen. Oxygen and iron easily induce oxidative stress in cases of pathologic conditions, such as iron excess [2–4], inflammation, and reperfusion [5,6].

In 1982, Okada and Midorikawa discovered ferric nitritotriacetate (Fe-NTA)-induced renal carcinogenesis model in rodents with serendipity [5,7–10]. The important three points are that (1) this model is faithfully reproducible, starting from the acute renotoxic phase [11,12], where numerous oxidative modifications in biomolecules are identified [13–19], to chronic iron excess phase [5,20]; (2) we can use wild-type rats; in the case of mice, we have to use Mutyh knockout mice because C57BL/6 strain used for knockout studies is generally cancer-resistant [21,22]; (3) this model suggests that carcinogenesis, at least some of them, can be iron addiction with ferroptosis-resistance [6,23]. Previously we reported that genomic alterations in Fe-NTA-induced rat renal cell carcinoma (RCC) as seen by array-based comparative genome hybridisation (aCGH) is prominent and is most similar to those of RCCs among human cancers [24]. Recently, we reported that the genomic alterations of Fe-NTA RCC in Mutyh knockout mouse are significantly less prominent than the rat counterparts [22]. However, nobody has thus far performed the cross-sectional comparison among murine, rat, and human RCCs on the basis of aCGH. In the present study, to
observe the species difference in oxidative stress-induced carcinogenesis and to obtain an insight regarding the characteristics of each species, we analysed the genomic alteration of RCCs among the three species in special reference to oxidative stress as etiology. We found a clear species difference, especially in the gain/loss ratio in the genome of RCCs. The implications of the results would be discussed.

Materials and methods

Comparison of the chromosomal alterations in RCCs among mice, rats, and humans

To comparatively analyse the patterns of the chromosomal alterations in RCCs of mice, rats, and humans, we used aCGH data of RCC samples from each species of those mammals. Regarding mice and rats, aCGH data from our own Fe-NTA-induced renal carcinogenesis experiments (GSE99535 and GSE636101) was used. Because we had used Agilent microarray products for our aCGH analyses [24], we also selected publicly available human aCGH data generated with Agilent microarrays, for comparison with a unified algorithm. We used aCGH data of clear cell renal cell carcinomas (CCRCCs) with metastasis in GSE43477 data set [25] and that of papillary renal cell carcinomas (PRCCs) in GSE63003 data set [26]. Agilent 60-mer oligonucleotide microarrays with 44, 180, and 185 k probes were used for the data sets (44 k for GSE43477, 180 k for GSE63003 and GSE99535, 185 k for GSE636101).

To compare the scales of the chromosomal alterations in RCC genomes among mice, rats, and humans, we calculated the percentage of chromosomal sites with a copy number aberration among all the sites in the whole genome as the quantitative measure. The copy number aberration frequency was calculated from the results of each aCGH analysis according to the following procedure: (1) compute a moving average of the signal log2 ratios for the CGH microarray probes distributed within 500 kbp from each point at every 100 kb along the chromosomes (limited to the autosomes); (2) plot values of the moving averages of the signal log2 ratios from the whole genome as a histogram and a genomic variation plot, and determine the thresholds on both sides, beyond which, the copy number aberration can be called via a visual evaluation of the histogram and the variation plot for each aCGH result; and (3) calculate the fraction of the chromosomal sites at which the copy number aberration was called according to the above defined thresholds.

We practically omitted some of the array-CGH data from the comparison analysis because the background noise levels were too high. According to the criterion, two of the 13 primary rat RCCs in GSE636101, seven of the 20 primary human CCRCCs in GSE43477 and four of the nine human PRCCs in GSE63003 were omitted from the analysis. Figure S1 shows genomic profiles of the aCGH data, plots of the moving averages of the signal log2 ratio values calculated along the autosomes of each species. The copy number aberration frequency is equivalent to the fraction of genomic sites, which corresponds to red points in each plot in Figure S1.

Results

Different patterns of chromosomal alterations in the genome of RCCs among mice, rats, and humans

We evaluated the similarities of deviation of chromosomal structures in the genome of RCCs among mice, rats, and humans.

| Sample name      | Number of normal loci | Number of deleted loci | Number of amplified loci | Fraction of copy number aberrations (%) |
|------------------|-----------------------|------------------------|--------------------------|-----------------------------------------|
| Murine RCC (total 23935 sites) | 21250 | 2674 | 11 | 11.22 |
| KO_1080          |                   |                       |                          |                                         |
| KO_1134          | 15314 | 8376 | 245 | 36.02 |
| KO_1097          | 19242 | 4679 | 14  | 19.61 |
| WT_1179          | 18916 | 3697 | 1322| 20.97 |
| Mean             | 18680.5 | 4856.5 | 398 | 21.95 |
| Rat RCC (total 25503 sites) |                   |                       |                          |                                         |
| FB7-1            | 19884 | 5509 | 110 | 22.03 |
| FB7-4            | 19836 | 4551 | 1116| 22.22 |
| FB7-7            | 19702 | 3717 | 2084| 22.75 |
| FB59-1           | 15654 | 9844 | 5   | 38.62 |
| FB14-3           | 16244 | 7643 | 1616| 26.96 |
| BF51-1           | 14756 | 9023 | 1544| 42.14 |
| FB14-6           | 18628 | 7688 | 9788| 52.87 |
| FB21-2           | 18523 | 5961 | 1788| 29.34 |
| FB45-4           | 19171 | 5939 | 193 | 37.59 |
| FB30-5           | 18021 | 5610 | 1872| 29.34 |
| BF57-5           | 18216 | 5846 | 1441| 28.57 |
| Mean             | 17761.9 | 6669.5 | 1071.6| 30.35 |
| Human CCRCC (total 26778 sites) |                   |                       |                          |                                         |
| M3P              | 21955 | 3155 | 1668| 18.01 |
| M4P              | 18465 | 5248 | 3065| 31.04 |
| M7P              | 21251 | 6888 | 2639| 20.64 |
| M9P              | 14753 | 4453 | 7572| 44.91 |
| M11P             | 19233 | 3706 | 3839| 28.18 |
| M12P             | 17973 | 7382 | 973 | 32.88 |
| M14P             | 10062 | 5678 | 11038| 62.42 |
| M15P             | 25128 | 733  | 917 | 6.16  |
| M16P             | 22590 | 2607 | 1581| 15.64 |
| M17P             | 18500 | 4470 | 3808| 20.91 |
| M18P             | 20201 | 3459 | 3118| 24.56 |
| M20P             | 20286 | 5722 | 770 | 24.24 |
| Mean             | 19199.8 | 4162.6 | 3415.7| 28.3  |
| Human PRCC (total 26945 sites) |                   |                       |                          |                                         |
| X2_prCC1         | 17928 | 19  | 8998| 33.46 |
| X13_prCC1        | 23224 | 372 | 3349| 13.81 |
| X15_prCC1        | 19478 | 3205| 4162| 22.73 |
| X18_prCC2        | 19895 | 2120| 4030| 26.16 |
| X36_uRCC         | 19063 | 647 | 7235| 29.25 |
| Mean             | 19917.6 | 1292.6 | 5734.8| 26.08 |

RCC: renal cell carcinoma; CCRCC: clear cell renal cell carcinoma; PRCC: papillary renal cell carcinoma.

Refer to ref. [22] for the details of murine and rat RCCs.
rats, and humans. Essentially, we compared our aCGH data from murine and rat RCC samples in Fe-NTA-induced renal carcinogenesis model with that from human RCC samples in the public database. The comparison analysis was based on the copy number aberration frequency, calculated from the distributions of the signal log2 ratios in each aCGH result. Overall, the copy number aberration was the most frequent in the rat RCCs and was the least frequent in the murine RCCs (Table 1 and Figure 1). Indeed, the copy number aberration frequency in the rat RCCs was closer to that of human RCCs than to murine RCCs. The average deviated fraction of genomic segments out of 44–185 k, either loss or gain, from the standard biallelic position was 21.95% (N = 4), 30.35% (N = 11), 28.30% (N = 12), and 26.08% (N = 5), respectively, for murine RCC, rat RCC, human CCRCC, and human PRCC. Notably, gain/loss ratio was remarkably different as indicated by 0.0820, 0.161, 0.821, and 4.44, respectively.

Discussion
Three distinct species in mammal (mice, rats, and humans) present RCCs of similar histology derived from renal tubules [10,22,27], which were analysed in the present study. We have compared the global genomic alterations of these RCCs in search of detectable amplification/deletion with aCGH. Strikingly, human RCCs revealed significantly higher amplification/deletion ratio than the rodent RCCs, and rat RCCs showed higher amplification/deletion ratio in the genomic alterations than murine RCCs. The genome with deletions would be more rapidly replicated than that with amplifications due to the physical length of DNA double-strands, hypothesising that the catalytic activity of DNA polymerase is similar for each species. Renal tubular cells are essential for daily metabolism to reabsorb important small molecules, such as glucose, amino acids, and cations/anions, after glomerular filtration. Thus, longer time required for DNA replication may be a critical burden for the precancerous cells containing amplifications in the genome to proliferate faster than the other cells during carcinogenesis. In contrast, cells with deletions should be easier to replicate. Interestingly, cellular size of renal tubules is almost the same among mice, rats, and humans. In this sense, the size of the organ, here cell numbers, may be a determinant in that larger organ

![Figure 1. Bar charts of the frequencies of genomic sites with a normal or aberrated copy number in each aCGH profile of RCCs from three distinct mammalian species (mouse, rat, and human). This figure corresponds to Table 1. Blue, orange, and red bars indicate fractions of the normal, deleted, and amplified genomic sites, respectively. Refer to the online version for colour.](image-url)
allows more room for more complicated processes out of physiological function in a larger population of cells. In general, larger organisms have bigger and slowly dividing cells with lower energy turnover, thus reducing the risk of cancer initiation during the long lifetime [28].

Regarding the total frequency of the genomic alterations, there was a tendency of less deviations from the biallelic standard position in the RCCs of mice than in those of rats or humans. This may be associated with a lower incidence of amplification as described above. Indeed, rat RCCs are more similar to human RCCs, though we need further studies on RCCs of other rodent models with different etiologies. This may lead to a general concern over the selection of the species for preclinical studies. In a recent study, we reported that these murine RCCs do not have homozygous deletion of p16^INK4A tumour suppressor genes nor methylation of the promoter region of the same gene [22]. These may indicate that using a murine model alone is risky when we consider the application of the preclinical results to humans. At least for the carcinogenesis of RCCs, rats are much closer to humans than mice. Thus, rat models, whether genetically engineered or not, need more attention from the researchers.

Lastly, there is a limitation in the present discussion in that etiology is usually difficult to be identified in the human spontaneous RCCs whereas etiology is oxidative stress via excess iron here in the rodent RCCs. Another limitation is that we used genetically engineered animals only for mice. This was indispensable in the present study because wild-type C57BL/6 background is cancer-resistant [22]. However, we will seek for different mouse strains with higher susceptibility of RCCs in the near future. Different average lifetime among species might be a determinant in that it is ~2 years for mice but >3 years for rats. At last, we have not used next generation sequencing yet, thus ignoring point mutations here.

In conclusion, the present results suggest that rat RCC models are more similar to actual human diseases than murine models in the light of genomic alterations, though there are several limitations. Further studies are in progress in our laboratory to answer the remaining questions.

Disclosure statement
No potential conflict of interest was reported by the authors.

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