NRF2 Intensifies Host Defense Systems to Prevent Lung Carcinogenesis, but After Tumor Initiation Accelerates Malignant Cell Growth

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Abstract

Nrf2 activation promotes resistance to chemical carcinogenesis in animal models, but activating mutations in Nrf2 also confer malignant characters to human cells by activating antioxidative/detoxifying enzymes and metabolic reprogramming. In this study, we examined how these contradictory activities of Nrf2, cancer chemoprevention and cancer cell growth enhancement, can be reconciled in an established mouse model of urethane-induced lung carcinogenesis. Using Keap1-knockdown (kd) mice, which express high levels of Nrf2, we found that urethane was rapidly excreted into the urine, consistent with an upregulation in the expression of urethane detoxification genes. Consequently, urethane-induced tumors were significantly smaller and less frequent in Keap1-kd mice than in wild-type mice. In contrast, tumor cells derived from Keap1-kd mice and transplanted into nude mice exhibited higher tumorigenicity compared with cells derived from wild-type mice. To identify the factors contributing to the tumor growth phenotype in the transplantation model, we performed a microarray analysis and found that many antioxidative stress genes were upregulated in the Keap1-kd–derived tumors. Therefore, we suggest that Nrf2 activation in cancer cells enhances their tumorigenicity, but global Nrf2 activation, as in Keap1-kd mice, simultaneously enhances antitumor immunity, thereby suppressing the growth potential of Keap1-kd tumors. Our findings provide relevant insight into the dual role of Nrf2 in cancer and warrant further studies of Nrf2 function during different stages of carcinogenesis. Cancer Res; 76(10); 3088-96. © 2016 AACR.

Introduction

The transcription factor Nrf2 plays important roles in the protective response against environmental stresses, particularly against oxidative and electrophilic insults (1, 2). In unstressed conditions, Nrf2 is bound by Keap1 and subjected to degradation through the ubiquitin–proteasome pathway. Upon exposure to oxidative or electrophilic stresses, reactive cysteine residues of Keap1 are chemically modified. Thereafter, the Keap1-mediated degradation of Nrf2 is eliminated, leading to Nrf2 accumulation in the nucleus. Subsequently, Nrf2 dimerizes with one of the small Maf proteins (sMaf) and binds to the specific DNA sequence referred to as antioxidant/electrophile response element, through which a variety of target genes, such as NAD(P)H quinone oxido-reductase (Nqo1), heme oxygenase 1 (Ho-1), and glutamate-cysteine ligase catalytic subunit (Gcl), are induced. These cytoprotective enzymes contribute to the cellular protection against oxidative and electrophilic insults.

Urethane (ethyl carbamate) is a prototypic carcinogen that induces lung adenoma and adenocarcinoma (3). Upon administration of urethane to mice, adenomas often develop in the lung, which later give rise to adenocarcinomas (4). Cytochrome P450 2E1 (Cyp2e1)-mediated oxidation converts urethane into vinyl carbamate epoxide (VCE), which serves as a potent carcinogen (5). VCE is converted into 1, 2-dihydroxyethyl carbamate by microsomal epoxide hydrolase (mEH), and subsequently the product is subject to conjugation with glutathione (6). As the mEH and Gstp1/p2 genes are targets of NRF2 (1, 7, 8), the detoxification pathway of urethane appears to be under the influence of Nrf2 activity. Many studies have demonstrated that Nrf2-deficient mice are susceptible to a variety of carcinogens (9–12). In contrast, Nrf2 activation in cancer cells has also been shown to contribute to the promotion of tumor growth in many forms of cancer (13, 14). These two rather contradictory aspects of Nrf2 function have been referred to as the “Double-Edged Sword of Nrf2” (15, 16). We have previously demonstrated that Nrf2 activity exhibits bidirectional stage-specific effects in urethane-induced lung carcinogenesis (17). Specifically, Nrf2-deficient mice exhibited more abundant microtumor nodules than wild-type mice at early stages (4–6 weeks) after urethane administration. In contrast, in the later stages (16 weeks after urethane treatment), wild-type mice showed large, malignant lung tumors with...
increased Nrf2 accumulation, whereas Nrf2-deficient mice rarely developed such malignant cancers (17). These results indicate that Nrf2 prevents cancer initiation in the early stages, whereas Nrf2 accelerates cancer progression in the advanced stages of urethane-induced lung carcinogenesis.

Given the above results from the Nrf2-deficient mice, we next wanted to address whether constitutive Nrf2 activation affects cancer incidence and malignancy. To this end, we exploited Keap1-knockdown (Keap1-kd) mice that show constitutive Nrf2 accumulation due to a systemic decrease in Keap1 expression (18). The Keap1-kd mice survive to adulthood and exhibit resistance against oxidative and electrophilic insults (19, 20). Therefore, the Keap1-kd mice serve as an excellent model of genetic Nrf2 induction.

Considering that Nrf2-deficient mice show increased susceptibility to urethane and develop many micronodules in the early stage (17), we hypothesized that Keap1-kd mice could be resistance against urethane-induced lung carcinogenesis, due to the constitutive activation of stress-responsive genes. To address this hypothesis, we utilized the urethane carcinogenesis model in Keap1-kd mice. We found that the Keap1-kd mice developed a significantly lower number of urethane-induced lung tumors than the wild-type mice. In contrast, when transplanted into nude mice, the Keap1-kd mice–derived tumor cells showed more vigorous growth than the wild-type mice–derived tumor cells. These results demonstrate that systemic activation of Nrf2 prevents urethane-induced lung carcinogenesis, whereas Nrf2 activation confers tumorigenicity on the cancer cells.

Materials and Methods

Experimental animals

Keap1-kd mice (5–9 weeks) and age-matched Keap1-wt mice with ICR genetic background were used (18, 21). The mice were maintained in a facility free of specific pathogens. Nude mice (8–9 weeks) were purchased from CLEA Japan. We mainly used male mice in this study to exclude gender biases. All animal experiments were performed under the approval of the Tohoku University Animal Care Committee.

Transplantation of tumors into nude mice

Urethane-induced lung tumors of approximately equal sizes (φ = 0.5–1.5 mm) from Keap1-kd and Keap1-wt mice were purchased from CLEA Japan. We mainly used male mice in this study to exclude gender biases. All animal experiments were performed under the approval of the Tohoku University Animal Care Committee.

Quantitative RT-PCR

Total RNA was extracted from the tissues using ISOGEN (Nippon Gene). First-strand cDNA was synthesized from the total RNA using random hexamers and Superscript III Reverse-Transcriptase (Invitrogen). Real-time RT-PCR was performed using 2X SYBR Green PCR Master Mix (Invitrogen) and the ABI PRISM 7300 sequence detector system (PE-Applied Biosystems). Sequences of primers and TaqMan probes were described previously (17). Following primers were newly prepared: Ppargc1a: forward ACAAGCTTTCGCGTGGATG, reverse TCTGTTGAGACCGCTAGCAA. Catalase: forward GCTTGAAGTGGTGAATGCG, reverse GCCAATTTTTGGATTGGCG, and TaqMan probe AAGGCCAGCCTAATGGCAAG.

Urethane-induced lung carcinogenesis experiments

Urethane (ethyl carbamate; 1 g/kg body weight) was intraperitoneally administered. At 8 weeks, 16 weeks, and 8 months after the administration, the mice were euthanized. The lungs were dissected and the total number of lung surface tumors was counted macroscopically. The diameter of the tumors was measured using an electronic caliper.

Gene expression analysis

Surface lung tumors were dissected, and surrounding tissues were carefully removed using a stereoscopic microscope. Tumors and nontumor tissues in lungs of urethane-administered mouse were pooled and subjected to a whole-mouse genome microarray analysis (4 × 44 k; Agilent Technologies). Expression array data were analyzed with GeneSpring software (Silicon Genetics). Heatmaps were generated utilizing Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and JAVA Treeview 159 (http://jtreeview.sourceforge.net/). The classifications of the selected genes according to their biologic and toxicologic functions were performed using Ingenuity Pathway Analysis (IPA) software (Ingenuity system). P value, represented as the negative log ratio of the IPA results, is calculated by the Fisher exact test.

Statistical analyses

The data are described as the mean ± SD. The statistical significance in differences was calculated by the Student t test or the Mann–Whitney U test. The values for the incidence of lung tumors were analyzed with the Fisher exact probability test. P values < 0.05 were considered significant.

Results

Keap1-kd mice express Nrf2 and its target genes at high levels

Urethane acquires carcinogenic activity through conversion into the electrophilic metabolite, vinyl carbamate epoxide (VCE; Fig. 1A). We previously found that intraperitoneal injection of urethane (1 g/kg body weight) increased Nrf2 protein level and expression of Nrf2 target genes in the lung of Keap1-wt mice (17). It has previously been shown that Keap1-kd mice are highly resistant to the damaging effects of oxidants due to the high expression level of Nrf2’s cytoprotective target genes (19, 20). In this study, we found that a number of genes that directly participate in the detoxification of urethane, that is, Cyp2e1, mEH, Gstp1, and Gstp2, were induced in the lung of Keap1-kd mice compared with Keap1-wt mice (Fig. 1B). In the liver, mRNA expression of mEH and Gstp2 was also higher in Keap1-kd mice when compared with Keap1-wt mice (Supplementary Fig. 1). These results indicate that the expression of urethane detoxification genes is enhanced in Keap1-kd mice.

The constitutive activation of the urethane detoxification system in Keap1-kd mice suggests that urethane is efficiently detoxified in Keap1-kd mice. It has previously been reported that urethane metabolites are mainly excreted into the urine (23). Therefore, we examined the concentration of urethane and its metabolite in plasma and urine of Keap1-kd and Keap1-wt mice after intraperitoneal injection of urethane. We collected plasma and urine samples one day after the injection of urethane (1 g/kg body weight) and subjected them to LC/MS-MS analyses. A selected reaction monitoring (SRM)
chromatogram of urethane and its positive control urethane-d5 (deuterium-labeled urethane as an internal standard) in plasma are shown in Fig. 1C and a SRM chromatogram of VCE and urethane-d5 in urine are shown in Fig. 1D.

The urethane level in the plasma was significantly lower in Keap1-kd mice compared with Keap1-wt mice one day after the urethane injection (Fig. 1E). Similarly, the level of VCE in the urine was also lower in the Keap1-kd mice than in the Keap1-wt mice (Fig. 1F). These results indicate that urethane and its metabolite VCE is efficiently detoxified and excreted by Keap1-kd mice, presumably by virtue of the enhanced activity of the urethane detoxification pathway.

Keap1-kd mice are resistant against urethane-induced tumorigenesis

To address the question of whether the susceptibility to urethane is altered in Keap1-kd mice, we applied the urethane-induced carcinogenesis methodology to the Keap1-kd and Keap1-wt mice. We analyzed the effects of urethane treatment under four different experimental conditions: short-term observation (8 weeks), mid-term observation (16 weeks), long-term observation (8 months), and long-term observation (8 months) with multiple administrations of urethane.

We found that in the short-term observation after a single urethane administration, 100.0% (4/4) of the urethane-treated Keap1-wt mice developed macroscopic (> 0.5 mm) lung...
surface tumors, compared with only 20% (2/10) of the Keap1-kd mice (φ > 0.5 mm; Fig. 2A and B; Table 1A). This result suggests that the induction of Nrf2 in Keap1-kd mice prevents urethane-induced lung tumorigenesis during the early phase.

We extended the observation time and examined the total number and diameter of lung surface tumors 16 weeks after urethane administration. The total number of lung surface tumors (φ > 1.0 mm) per mouse was significantly increased during this extended period in the Keap1-wt mice (Fig. 2C and D; Table 1B). There also exists the possibility that Keap1-kd tumor cells may grow vigorously during the later stages of the urethane-induced tumorigenesis, as Nrf2 shows potent oncogenic activity in many types of human cancers (13, 22). To examine this possibility, we extended the observation term again and examined the total number and diameter of urethane-induced tumors 8 months after a single administration of urethane. The total number of surface tumors (φ > 1.0 mm) per mouse was significantly increased in the Keap1-wt mice at 8 months (Fig. 3A and B; Table 1C). In contrast, the numbers of surface tumors did not significantly increase in the Keap1-kd mice even at this late time point (Fig. 3A and B; Table 1C). By determining the average diameter of all of the tumors in both genotypes, we found that Keap1-kd mice also developed much smaller tumors when compared with the Keap1-wt mice (Fig. 3C). These results indicate that constitutive induction of Nrf2 through the knockdown of Keap1 expression strongly restricts the growth of urethane-induced lung tumors.

To examine whether an increased amount of urethane and its metabolites could increase urethane-mediated tumorigenesis in Keap1-kd mice, we conducted a tumorigenesis experiment using multiple injections of urethane. Both Keap1-kd and Keap1-wt mice were injected weekly with urethane for four consecutive weeks, subsequently the mice were examined 8 months after the first urethane administration (Fig. 3D). Surprisingly, the initiation of tumorigenesis was not dramatically enhanced by this treatment, and the number of surface tumors was still significantly lower in Keap1-kd mice than in Keap1-wt mice (Fig. 3E; Table 1D). Interestingly, using this experimental procedure, the diameter of Keap1-kd tumors became similar to that of Keap1-wt mice (Fig. 3F). In addition, Keap1-kd tumors contained an increased number of Ki67-positive cells compared with similar sized Keap1-wt tumors (top panels in the Supplementary Fig. S2A), whereas the tumor bearing Keap1-kd lung tissue harbors a higher number of infiltrating inflammatory cells than the Keap1-wt lung (bottom panels in the Supplementary Fig. S2B). These results indicate that the Keap1-kd tumors have a high tumorigenic potential, but the increased number of inflammatory cells might attenuate the tumor growth in the Keap1-kd lung tissue.

Cancer-resistant host microenvironment is activated in Keap1-kd mice

We previously found that systemic Nrf2 activation in Keap1-kd mice generates a cancer-resistant host immune environment by attenuating the activity of myeloid-derived suppressor cells (MDSC), a potent cancer immunosurveillance cell type (24). Intracellular accumulation of reactive oxygen species (ROS) in MDSCs (ROS-in-MDSC) leads to the suppression of CD8+ T-cell–mediated cancer immunity, hence the ROS level serves as a good indicator of MDSCs activity (24, 25). Therefore, we hypothesized that an increase in Nrf2 activity will decrease the ROS-in-MDSC level in tumor-bearing Keap1-kd mice, thereby attenuating the activity of MDSCs, leading to the generation of a cancer-resistant host immune environment in Keap1-kd mice.

To test this hypothesis, we conducted tumor cell transplantation studies into immunodeficient nude mice, a gold standard experiment to evaluate the tumorigenicity of cancer cells without the influence of host-environment interactions (26). We dissected lung tumors of approximately equal sizes (φ = 0.5–1.5 mm) from the Keap1-wt and Keap1-kd mice 8 months after a single urethane administration, transplanted them into nude mice, and evaluated their tumorigenicity. Notably, during the 5-month observation period, tumor cells from Keap1-kd mice grew much more aggressively than tumor cells from Keap1-wt mice.
Keap1-wt mice. Representative tumors taken from the back of nude mice are shown in Fig. 4A, and the sizes of the tumors measured every month are depicted in Fig. 4B. These results indicate that the tumor cells derived from Keap1-kd mice are more highly proliferative compared with those from the wild-type mice when transplanted into an immunodeficient host environment.

Expression profile of cancer-related genes in Keap1-kd tumor cells

To explore the mechanisms underlying the enhanced growth of Keap1-kd cancer cells in nude mice, we conducted expression microarray analysis and compared the gene expression profile between Keap1-kd and Keap1-wt tumor cells. For this purpose, we extracted total RNA from tumors and non-cancerous normal tissue from both Keap1-kd and Keap1-wt mice 8 months after a single administration of urethane. We selected genes that were induced more than 2-fold in the tumors relative to normal lung tissue in both genotypes of mice (Fig. 5A). A set of 566 genes were found to be commonly upregulated in both the Keap1-kd and Keap1-wt cancer tissues, including genes that regulate lung development, such as Sox9, Id2, and Foxa2 (data not shown). These three genes have previously been shown to participate in lung cancer progression under the regulation of Nrf2 (17). A distinct set of 489 genes was exclusively upregulated in the Keap1-kd tumors. Employing IPA analysis, we identified 20 downstream genes responsible for the enhanced growth of Keap1-kd tumors and confirmed their differential expression patterns between the Keap1-wt and the Keap1-kd tumors (Fig. 5B). Most of the genes encode antioxidant and detoxification enzymes, which are well-known downstream target genes of Nrf2 (Supplementary Table S1; refs. 27–31). Of the 20 genes, Glutathione peroxidase 2 (Gpx2), Catalase (Cat), Ppara1A, Glutathione-S-transferase a4 (Gsta4), and Glutathione reductase (Gsr) were found to be highly expressed in the Keap1-kd cancers in comparison with the Keap1-wt cancers (pink dots in Fig. 5B), and all five of these genes have been reported to contribute to cancer cell proliferation through eliminating cellular ROS level (32–35). We confirmed this change in gene expression by means of manual quantitative RT-PCR (Fig. 5C). In addition, we noticed an increase in Multidrug resistance protein 3 (Mtrp3) expression, which contributes to cellular multidrug resistance (36). These results suggest that the Keap1-kd cancer cells retain higher level of drug resistance than do the Keap1-wt cancer cells.

However, it should be noted that the tumors in Keap1-kd mice were all small, and that the cancer cells from the Keap1-kd mice only proliferated vigorously in the micro-environment of the nude mouse. Therefore, these results demonstrate that, although increased expression of antioxidant genes contributes to the enhanced proliferation of Keap1-kd cancer cells, the proliferation of these tumors is severely repressed by the anticancer immunity mediated by the global increase in Nrf2 activity in Keap1-kd mice (summarized in Fig. 6).
Discussion

It is widely accepted that Nrf2 attenuates toxicities of many oncogenic compounds by inducing the expression of a series of detoxifying and antioxidative stress enzyme genes (1). For example, urethane treatment induces Nrf2 accumulation, and the subsequent induction of detoxifying and antioxidative stress enzymes alleviates the initiation of lung cancers in wild-type mice (17). In this study, we have demonstrated that Keap1-kd mice, which express cytoprotective enzymes at a high level, are significantly resistant to urethane-induced lung carcinogenesis, indicating that the cytoprotective enzymes regulated by Nrf2 are crucial for the prevention of urethane-induced carcinogenesis. Intriguingly, while the number and size of urethane-induced tumors were significantly decreased in the Keap1-kd mice, tumor cells derived from the Keap1-kd mice grew much more vigorously upon transplantation into nude mice than the wild-type mouse–derived cancer cells. These results demonstrate that, while the Keap1-kd mouse–derived cancer cells acquire a strong cue for malignant transformation, their proliferation ability is severely repressed by the anticancer immunity mediated by the global increase in Nrf2 activity in these mice.

Figure 3.
Long-term urethane-induced lung carcinogenesis. A, experimental protocol for one-shot urethane administration. Mouse lungs were examined 8 months after urethane administration. Representative gross observations of surface lung tumors in Keap1-wt and Keap1-kd mice are depicted. Arrowheads, the surface tumors. Scale bar, 10 mm. Representative hematoxylin and eosin–stained sections (bottom). Scale bar, 40 μm. B, number of surface lung tumors (φ > 1 mm) in Keap1-wt (n = 7) and Keap1-kd (n = 8) mice. The color of the dots indicates the size of the largest tumor in each individual mouse of both genotypes. Each dot represents the total number of macroscopic tumors (φ > 0.5 mm) in an individual mouse. The color of the dots indicates the size of the largest tumor in each mouse lung. C, average tumor diameter in each genotype of mouse. D, experimental protocol for the four consecutive urethane administration experiment. Mouse lungs are examined 8 months after four consecutive administrations of urethane. Representative gross observations of surface lung tumors in Keap1-wt and Keap1-kd mice. Arrowheads, the surface tumor. Scale bar, 10 mm. Representative hematoxylin and eosin–stained sections (bottom). Scale bar, 40 μm. E, number of surface lung tumors (φ > 1 mm) in Keap1-wt (n = 5) and Keap1-kd (n = 7) mice. The color of the dots indicates size of the largest tumor in each individual mouse of both genotypes. Each dot represents total number of macroscopic tumors (φ >1.0 mm) in individual mouse. The color of dots indicates size of the largest tumor in each mouse lung. F, average tumor diameter in each genotype of mouse. The data are presented as the mean ± SD. The significant differences by Student t test are indicated (*, P < 0.05; **, P < 0.01).

Figure 4.
Keap1-kd mice tumors transplanted into nude mice grow larger than that of Keap1-wt mice. A, gross observations of tumors transplanted in nude mice. Scale bar, 0.5 mm. Representative hematoxylin and eosin–stained sections (bottom). Scale bar, 20 μm. B, growth curve of Keap1-wt and Keap1-kd tumors transplanted in nude mice. The data are presented as the mean ± SEM. The statistically significant differences by Mann–Whitney unpaired U test are depicted (*, P < 0.05; n = 6–8 in each group).
Keap1-kd mice. Consequently, the size of the tumors is kept small in the Keap1-kd mice.

It has been shown that the immunosuppressive activity by MDSCs is primarily regulated by the intracellular ROS level (37) and that the Nrf2-mediated antioxidant system appears to play a crucial role for the reduction of immunosuppressive activity in MDSCs (24, 38). Recently, \textit{ex vivo} experiment of bone marrow–derived macrophage using \textit{Nrf2}–deficient mice showed that Nrf2 contributes to CD8\textsuperscript{+} T-cell function by regulating \textit{g-GCS} and \textit{xCT} (39). We recently reported that the immune microenvironment of Keap1-kd mice leads to resistance against metastasis of lung cancer cells and that activation of Nrf2 by chemical inducers reduces ROS levels in MDSCs, which in turn strengthens host immunity against metastatic cancer cells (38). We found that when Keap1-kd tumors were transplanted into T-cell–deficient nude mice, they proliferated vigorously, supporting the notion that Nrf2-mediated enhancement of anticancer immunity is critically important to regulate the cancer-resistant host microenvironment. We would postulate that, the genetic induction of Nrf2 by knockdown of the \textit{Keap}1 gene results in reduced MDSCs activity, and consequently a reduction in both tumor number and size in response to urethane-induced carcinogenesis (summarized in Fig. 6).

To clarify the mechanisms underlying the strong proliferative activity of the transplanted Keap1-kd cells, we examined the gene expression signature of the Keap1-kd tumors. Our microarray analysis revealed that a battery of Nrf2 downstream genes is highly expressed in the Keap1-kd tumors. Particularly, expressions of \textit{Gpx2}, \textit{Gsr}, \textit{Cat}, \textit{Mrp3}, \textit{Gata2}, and \textit{Ppargc1A} genes, all of which have been reported to be regulated by Nrf2, are increased (27–31, 36, 40, 41). Of these antioxidant proteins, Catalase encoded by \textit{Cat} is known to play a crucial role in the ROS-scavenging system by converting hydrogen peroxide to water (31). A recent study revealed the contribution of Catalase to the enhancement of lung cancer malignancy by reducing ROS level (32). PPAR gamma coactivator-1\textalpha (\textit{Pgc1\textalpha}), a transcriptional coactivator encoded by \textit{Ppargc1A}, also plays important roles in cellular protection against ROS by increasing the expression of antioxidant enzymes (27). In addition, \textit{Pgc1\textalpha} has also been shown to be highly expressed in several types of epithelial cancer where it contributes to ROS scavenging (34). Given these previous reports, our current results indicate that constitutively activated Nrf2 in the Keap1-kd tumor cells contributes to malignant progression by reducing ROS levels through inducing the expression of multiple antioxidant genes. Similarly, treatment of tumor-bearing mice with antioxidants, such as N-acetylcysteine (NAC) and vitamin E, have been shown to reduce intracellular ROS levels and promote cancer progression.
in experimental carcinogenesis in multiple tissues (42). These wide-ranging observations suggest that antioxidants may exert accelerating effects on cancer progression at the late stages of carcinogenesis.

Constitutive activation of Nrf2 in Keap1-kd mice produces the same positive effect on the proliferation of tumor cells, but as Nrf2 concomitantly activates anticancer immunity, it does not promote unrestricted tumor growth. Thus, our results show that the systemic activation of Nrf2 prior to the administration of carcinogens prevents urethane-induced lung carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Satoh, T. Moriguchi, M. Yamamoto
Development of methodology: H. Satoh, D. Saigusa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Satoh, T. Moriguchi, D. Saigusa, L. Baird, L. Yu, T. Shibata
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Satoh, T. Moriguchi, D. Saigusa, L. Baird, L. Yu, T. Shibata, M. Yamamoto
Writing, reviewing, and/or revising of the manuscript: H. Satoh, T. Moriguchi, D. Saigusa, L. Baird, T. Shibata, M. Yamamoto

References

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