Black Raspberries Protectively Regulate Methylation of Wnt Pathway Genes in Precancerous Colon Tissue

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Abstract

Ulcerative colitis is frequently an intermediate step to colon cancer. The interleukin-10 knockout mouse is a genetic model of this progression. We report that knockout mice fed 5% black raspberries (BRB) had significantly less colonic ulceration as compared with knockout mice that consumed the control diet. Dysfunction of the Wnt signaling pathway is a key event in ulcerative colitis–associated colon carcinogenesis. Therefore, we investigated the effects of BRBs on the Wnt pathway and found that the BRB-fed knockout mice exhibited a significantly lower level of β-catenin nuclear translocation. We followed-up this observation by evaluating the effect of BRBs on selected Wnt pathway antagonists. The mRNA expression levels of wif1, sox17, and qki were diminished in the knockout mice, whereas they were expressed at normal levels in knockout mice that were fed BRBs. The lower mRNA expression of these genes in the colon from the knockout mice correlated with hypermethylation of their promoter regions; BRBs decreased their promoter methylation and increased mRNA expression of these genes. This hypomethylation was associated with elevated protein expression of key proteins/enzymes that augment methylation, for example, dnmt3b, hdac1, hdac2, and mbd2 in the knockout mice; in addition, BRBs decreased the protein expression of these proteins/enzymes. The knockout mouse model recapitulates what occurs in human ulcerative colitis. Promoter methylation of CDH1 and SFRP1 was significantly higher in human ulcerative colitis tissues compared with their adjacent normal tissues. In conclusion, our results suggest that BRBs inhibit colonic ulceration and, ultimately, colon cancer partly through inhibiting aberrant epigenetic events that dysregulate Wnt signaling. Cancer Prev Res; 6(12): 1317–27. ©2013 AACR.

Introduction

The Wnt signaling pathway promotes increased nuclear localization of β-catenin protein when associated with T-cell factor (TCF) and other proteins, and participates in initiating the transcription of cancer-associated genes. In the normal colon epithelial cells, much of the β-catenin is bound to E-cadherin at the cellular membrane where it participates in cellular adhesion and differentiation processes. β-Catenin binds with APC, GSK3β, axin2, and CK1α proteins to prime its destruction by proteasome degradation and these processes keep β-catenin activity in check (1).

Dysfunction of Wnt signaling that occurs in more than 85% of sporadic colon cancer is primarily caused by mutation of the APC gene (2, 3). Ulcerative colitis is a precancerous disease that is caused by chronic inflammation. Ten percent of patients with ulcerative colitis progress to colon cancer within 20 years (4). Dysfunction of the Wnt to β-catenin signaling pathway is a key event in the genesis of ulcerative colitis–associated colon carcinogenesis (3). However, APC mutations are rarely observed in these cases; instead, Wnt signaling dysfunction in ulcerative colitis–associated colon cancer is strongly associated with epigenetic silencing of negative regulators of the pathway (3, 5, 6).

Although genetic mutations are generally irreversible, epigenetic alterations can be reversed by exogenous agents (7, 8), including some constituents of foods (9). We (10) have reported that black raspberries (BRB), which are rich in protective antioxidant and anti-inflammatory compounds such as anthocyanins and ellagitannins, inhibit colon carcinogenesis in animal models. In addition, we recently reported that a 5% BRB diet reduced colonic injury in a mouse model in which ulcerative colitis was induced by dextran sodium sulfate (DSS; ref. 11). This protective effect was associated with reduced levels of cytokines TNF and interleukin-1β (IL-1β), as well as COX-2 and prostaglandin E2 (PGE2) in colonic tissues (11). COX-2 is target of Wnt signaling (12), and we recently reported that patients with...
colon cancer who consumed BRBs exhibited hypomethylated inhibitor genes Wnt signaling pathway (13). Thus, BRBs affect carcinogenesis partially by regulating the level of DNA methylation of selected genes. For this study, we have used the IL-10 knockout mouse because it is a genetic animal model that recapitulates the genesis of human ulcerative colitis and ulcerative colitis–initiated colon cancer (14). We recently reported that the DNA (cytosine-5)-methyltransferase 1 (DNMT1) protein was reduced in colon tumor tissue collected from patients that consumed BRBs (13). We studied the effect of BRBs on other methylation-controlling enzymes and proteins. The DNMT3b protein was evaluated because it is involved in de novo DNA methylation, and has been shown to be overexpressed in colorectal cancer (15). In addition, we assessed the methylation binding domain 2 (MBD2), histone deacetylase 1 (HDAC1), and HDAC2, all of which are involved in gene silencing via chromatin condensation (16, 17), and we measured the relative levels of mRNA expression of the cognate genes.

Potential effects of the 5% BRB diet on the methylation status and mRNA expression of the selected regulator genes, wnt1, sox17, dkk2, dkk3, qki, and wnt3a, of the Wnt signaling pathway in colonic tissues from IL-10 knockout mice were also examined. Finally, we assessed the state of methylation of selected Wnt signaling regulatory genes in human ulcerative colitis specimens. It has been previously reported that the WNT regulatory genes, APC, APC2, SFRP1, and SFRP2 are hypermethylated in human ulcerative colitis specimens (18, 19). In the current study, we extended this database by evaluating cadherin-1 (CDH1; also called E-cadherin), WIF1, and WNT3A in colonic tissues from healthy donor as well as paired adjacent normal and ulcerative colitis tissues from patients with ulcerative colitis.

Overall, our results lend credence to the supposition that including significant quantities of BRBs in the diet may reduce the risk of patients with ulcerative colitis to develop colon cancer. In addition, our data suggest that part of the mechanism of prevention by BRBs is by antagonizing the development of inappropriate epigenetic events at the onset of cancer development.

Materials and Methods

Animals, berry treatment, and colon preparation

All protocols were carried out in accordance with institutional guidelines for animal care procedures as dictated by the Ohio State University Animal Care and Use Committee. Wild-type (WT) and knockout male mice were purchased from The Jackson Laboratory when they were 3 to 4 weeks old. The animals were placed in the protocol 1 week after they arrived from the vendor, and the study was of 8 weeks duration. WT mice were fed a control diet (n = 5) or control diet supplemented with 5% BRBs (n = 5), and knockout mice were fed either control diet (n = 15) or control diet supplemented with 5% BRBs (n = 15). The control diet was the American Institute of Nutrition synthetic diet 76A (AIN-76A; Dyets Inc). The preparation of BRB powder is detailed by Montrose and colleagues (11), and is also provided in Supplementary Materials; this is the same batch of BRB powder used by Montrose and colleagues (11). The mice were sacrificed by CO2 asphyxiation and full-length colons were removed, flushed with cold saline, and opened longitudinally. They were then Swiss-rolled, formalin-fixed and paraffin-embedded (FFPE), and stained with hematoxylin and eosin (H&E). The stained colonic tissues were examined according to standard pathologic criteria to confirm both normalcy and to demark regions of colonic ulceration.

Evaluation of colonic ulceration

FFPE colons were stained by H&E for colonic ulceration analysis. The entire colon was viewed under ×200 magnification (high-power view). The percentage of ulceration was calculated as the involved tissue in high power areas over total high power areas of the entire length of the colon. Areas of ulceration in the mucosa and submucosa were counted separately. Colons from all mice on this study were evaluated for colonic ulceration in a blinded manner by M. Yearsley.

Immunohistochemical staining and computer-assisted image analysis

Paraffin-embedded colonic tissues from the mice were cut into 4-μm sections and placed on slides. Staining procedures, antibody information for DNMT3B, MBD2, HDAC1, HDAC2, and β-catenin, and procedures for computer-assisted image analysis are detailed in Supplementary Materials.

Human colonic tissues

Forty-eight normal colon specimens from healthy donors, as well as 24 paired ulcerative colitis specimens and their adjacent normal tissues were obtained from the Cooperative Human Tissue Network (CHTN). The specimens were obtained and used in accordance with the dictates of the Institutional Review Board of the Medical College of Wisconsin (Milwaukee, WI).

Real-time PCR

mRNA was extracted from paraffin-embedded entire mouse colon tissues using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion). Two micrograms of total RNA per sample was reverse transcribed using SuperScript III RT (Invitrogen). Quantitative PCR (qPCR) procedures and primer information is provided in Supplementary Materials.

Pyrosequencing

Paraffin-embedded entire mouse colon tissues from 5 WT mice fed control diet, 5 WT mice fed BRB diet, 10 knockout mice fed control diet, and 10 knockout mice fed BRB diet were cut into 10-μm sections for DNA extraction. Approximately, 10 μg of each frozen human colonic tissue specimen, representing true normal (N), ulcerative colitis, or adjacent normal-appearing tissue (AN), was used for DNA extraction. Both mouse and human colonic DNAs were
extracted using PicoPure DNA kit (MDS Analytical Technologies). Extracted DNA was purified using the QIAquick PCR Purification Kit (Qiagen). Of note, 500 ng of extracted DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research). A pyrosequencing system (Qiagen) was used to quantify methylated CpGs as described previously (20). Specified primer sequences in the promoter region of the evaluated genes are listed in Supplementary Materials.

Statistical analysis

Data from evaluation of colonic ulceration, immunohistochemical staining, real-time PCR, and promoter methylation determined by pyrosequencing were compared by the Student t test. All analyses were two-sided, and a P value less than 0.05 was considered to be significant.

Results

Dietary BRBs reduce colon ulceration in IL-10 knockout mice

Representative H&E staining of colonic tissues from WT mice that consumed control or BRB diet, and knockout mice on either control or BRB diet are shown in Fig. 1A–D, top to bottom, respectively. Figure 1E depicts quantified results. The data show that knockout mice that consumed dietary BRBs exhibited significantly less ulceration in colonic mucosa and submucosa than did the knockout mice on control diet.

Effect of BRBs on β-catenin

The Wnt signaling cascade causes an increase in β-catenin protein translocation into the nucleus (1, 21). Representative staining of β-catenin protein in WT mice fed either control or BRB diet is depicted in Fig. 2A and B, respectively, whereas its appearance in the knockout mice is seen in Fig. 2C (control diet) and Fig. 2D (BRB diet). Quantification of the immunohistochemical staining is depicted in Fig. 2E. The percentage of stained nuclei within colonic tissues collected from WT mice that were fed either control or BRB-containing diets was statistically identical to that in adjacent normal-appearing tissues from knockout mice fed control or BRB diet. However, twice as many cells within the ulcerative colitis tissue from the knockout mice exhibited strong nuclear staining. In contrast, nuclear staining in the ulcerative colitis tissue from the knockout mice that consumed BRBs was not significantly different from the adjacent normal tissue. The mRNA expression level of β-catenin was found to be elevated in the knockout mice, but significantly less in the knockout mice that consumed BRBs (Fig. 2F).

BRBs modulate expression of Wnt signaling pathway regulators

mRNA expression of the Wnt signaling pathway negative regulators wif1, sox17, and qki in knockout mice fed control or 5% BRB diets was measured using real-time PCR. All these genes were expressed at lower levels in knockout mice that were fed the control diet as compared with the WT mice on either control or berry diet, and BRBs maintained their expression at control levels in the knockout mice (Fig. 3A–C, respectively). In addition, we evaluated the effects of BRBs on mRNA expression of dkk2 and dkk3 (Fig. 3D and E). Neither of these genes was downregulated in the knockout mice; however, animals on the BRB diet exhibited significantly higher mRNA expression levels of dkk2 and dkk3. Interestingly, BRBs decreased mRNA expression of wnt3a in the knockout mice (Fig. 3F).

We used pyrosequencing to evaluate promoter methylation of the same six genes, wif1, sox17, qki, dkk2, dkk3, and wnt3a, and the data are shown in Fig. 4A–F. In knockout mice on control diet, wif1, sox17, qki, and dkk2 were relatively hypermethylated than in WT mice on control or BRB diet, and BRBs maintained the percentage of methylation at control levels (Fig. 4A–D, respectively). In contrast, the level of methylation of dkk3 was not increased in the knockout mice, and BRBs had no effect on its methylation level (Fig. 4E).

In addition, we evaluated the promoter of Wnt ligand gene wnt3a. Wnt3a has been reported to be one of the most upregulated genes in the inflamed human colon (21). Therefore, we anticipated that the gene would be relatively hypomethylated in the knockout mice, and that BRBs would cause the level of methylation to be maintained at the control level. Instead, we found that wnt3a was significantly hypermethylated in the knockout mice, and BRBs cause the level of methylation to be maintained at control levels (Fig. 4F). As mentioned earlier, mRNA expression of wnt3a was decreased by BRBs in the knockout mice (Fig. 3F) although the berries decreased promoter methylation of wnt3a (Fig. 4F). These results suggest that other mechanisms are involved in the regulation of wnt3a expression.

Immunohistochemical quantification of DNMT3B, MBD2, HDAC1, and HDAC2

DNMT1 is chiefly responsible for maintaining DNA methylation homeostasis, whereas DNMT3B, HDAC1, HDAC2, and MBD2 are involved in de novo DNA hypermethylaton and chromatin methylation, and in converting euchromatin into heterochromatin (15–17, 22, 23). All of these enzymes have been shown to be overexpressed in several tumor types, including colorectal cancer (16). As noted previously (13), biopsies collected from human patients with colorectal cancer after they had consumed BRBs for an average of 4 weeks had lower levels of DNMT1 protein. Therefore, we quantified the levels of methylation-regulating proteins in the knockout mice to see whether BRBs affect their expression, and whether this might be associated with their ability to correct aberrant methylation. Immunohistochemical staining of DNMT3B, MBD2, HDAC1, and HDAC2 is depicted in Fig. 5E. The levels of all enzymes were significantly elevated in the ulcerative colitis samples from the knockout mice. However, specimens from the BRB-treated knockout mice exhibited levels that were statistically comparable with the normal colon from WT mice on control or berry diet (Fig. 5A–D). The mRNA expression of dnm3A, mbd2, hdac1, and hdac2 is
depicted in Supplementary Fig. S1. Knockout animals on the BRB diet exhibited significantly decreased expression of all of these mRNAs.

**Promoter methylation of the Wnt signaling pathway negative regulator genes is elevated in ulcerative colitis specimens from patients**

We and other researchers (8, 13) have shown that the level of methylation in the promoter regions of the Wnt signaling pathway negative regulatory genes SFRP2, SFRP5, and WIF1 are elevated in human colon tumor specimens. Dhir and colleagues (18) and You and colleagues (19) noted that APC, APC2, SFRP1, SFRP4, SFRP5, and DKK1 also become epigenetically silenced during the transition from normal to ulcerative colitis to human colon cancer. Therefore, we evaluated whether CDH1, SFRP1, WIF1, and APC are hypermethylated in ulcerative colitis tissues. The levels of methylation of CDH1 and SFRP1 were found to be significantly higher in the ulcerative lesions when compared with adjacent normal specimens (Fig. 6A and B, respectively). Interestingly, we did not observe significant differences in promoter methylation between tissues from healthy donors and the adjacent normal-appearing tissues from patients with ulcerative colitis for APC, WNT3A, and WIF1 (Fig. 6C–E, respectively). These levels correlated with increases of more than 50% in 14 of 24 patients for CDH1.
(Fig. 6A) and in 6 of 24 patients for SFRP1 (Fig. 6B). The percentage methylation value for the APC gene was very low in all specimens, and there was no significant difference among the specimens (Fig. 6C). However, when the inflamed tissues were compared with the adjacent normal-appearing tissue collected from the same patient, 9 of 24 lesions had a more than 50% increase in the level of hypermethylation of APC (Fig. 6C). The data for WNT3a also showed that the hypermethylation level of the gene in the ulcerative colitis tissues significantly exceeded that measured in the corresponding normal-appearing adjacent region, and a more than 50% increase in methylation was found in 5 of 24 patients (Fig. 6D). The methylation levels of WIFI were not significantly altered in ulcerative colitis compared with normal tissues from healthy donors and adjacent normal specimens (Fig. 6E).

Discussion

In the absence of a Wnt ligand, the β-catenin protein is primarily either bound to E-cadherin or within a complex
that augments its destruction by proteasome degradation (1). In normal cells, the binding of a Wnt ligand to Disheveled initiates the canonical Wnt signaling pathway, which results in the transcription of Wnt-specific genes that regulate cell-fate decisions. There is considerable regulation of the activity of the pathway through extracellular and intracellular controlling proteins. Dysfunction of this pathway is a formative event in the genesis of sporadic colon cancer, primarily by mutation or occasional hypermethylation of the APC gene (1). However, the genetic animal model used herein recapitulates the genesis of ulcerative colitis–associated colon cancer (14), and the molecular mechanism seems to be primarily epigenetic in nature (3, 5, 6).

β-Catenin–mediated signaling is a key regulator of epithelial proliferative responses, and it increases in repair responses in human chronic ulcerative colitis (24). Activation of β-catenin signaling increases in accordance with the degree of mucosal inflammation in human ulcerative colitis and one of the mechanisms that correlates with the genesis and maintenance of colitis is inappropriate nuclear accumulation of β-catenin (24). These observations are similar to those found in animal models of ulcerative colitis. For example, staining of phosphorylated-β-catenin increases in colitic and dysplastic colon of IL-10 knockout mice and is, therefore, suggested to be a biomarker of colitis-induced neoplastic transformation (24). In DSS-induced ulcerative colitis and cancer in mice, the β-catenin nuclear/cytoplasmic translocation is an early event in the development of dysplastic lesions (25). On the basis of these observations, increased activation of β-catenin signaling can lead to oncogenic transformation of colonic epithelium.

Wnt3a, a Wnt ligand gene, is associated with the regulation of Wnt pathway and repair, and it has been shown that activation of wnt3a signaling stimulates intestinal epithelial repair by promoting c-Myc–regulated gene expression (26). Furthermore, wnt3a is one of the most upregulated genes so far known in inflamed human colon (19). In the current study, we showed that BRBs decreased promoter methylation of wnt3a (Fig. 3F) and, interestingly, the berries also

Figure 3. Dietary BRBs protectively modulate mRNA expression of genes in the Wnt signaling pathway in the entire colon collected from IL-10 knockout (KO) mice. mRNA expression of (A) wif1, (B) sox17, (C) qki, (D) dkk2, (E) dkk3, and (F) wnt3a. *, P < 0.05.
decreased mRNA expression of \textit{wnt3a} (Fig. 4F) in colons from knockout mice, suggesting that they decrease activation of \textit{wnt3a} signaling. These results agree with the observation that BRBs decreased $\beta$-catenin nuclear localization (Fig. 2); BRBs effectively inhibited the translocation of this transcription-regulating cofactor. In addition, our results suggest other mechanisms are involved in regulating the expression of \textit{wnt3a}. Mesalamine is a mainstay therapeutic agent in human chronic ulcerative colitis; partly through decreasing $\beta$-catenin activation which, in turn, decreases colitis-induced dysplasia and colitis-associated cancer (24). Our findings suggest other mechanisms are involved in regulating the expression of \textit{wnt3a}.

Expression of $\beta$-catenin mRNA was found to be significantly elevated in knockout mice, and was reduced by berries in these mice. Upregulation of the gene in colon tissue of the knockout mice was unexpected. The mechanism causing increased $\beta$-catenin mRNA production and its downregulation by BRBs is unknown; however as discussed further, we speculate that it could be associated with the hypermethylation of \textit{qki}. Alternatively, it has been shown that Disheveled–KSRP complex stabilizes $\beta$-catenin mRNA (27).

Dhir and colleagues (18) noted that the Wnt pathway negative-regulatory genes, \textit{APC}, \textit{APC2}, \textit{SFRP1}, \textit{SFRP2}, \textit{SFRP4}, \textit{SFRP5}, and \textit{DKK1} become epigenetically down-regulated during the transition from normal human colon to ulcerative colitis to colon cancer. We investigated the regulatory genes \textit{wif1}, \textit{sox17}, \textit{dkk2}, \textit{dkk3}, and \textit{qki} in the IL-10

![Figure 4. Regulation of promoter methylation of Wnt signaling pathway genes by BRBs in whole colon specimens collected from IL-10 knockout (KO) mice. Promoter methylation of (A) \textit{wif1}, (B) \textit{sox17}, (C) \textit{qki}, (D) \textit{dkk2}, (E) \textit{dkk3}, and (F) \textit{wnt3a}. * P < 0.05.](image)
knockout mice to determine whether BRBs affect their levels of transcription and DNA methylation. Wif1, dkk2, and dkk3 are extracellular antagonists of Wnt ligands (28, 29), whereas sox17 augments degradation of the TCF–β-catenin complex (29, 30). The RNA-binding protein gene quaking (qki) was included in these investigations because its loss of expression is a common feature in human colon cancer. In addition, this gene controls the levels of β-catenin, presumably through interaction with its cognate protein with a response element in the β-catenin 3′-untranslated region (UTR; ref. 31). Wif1, sox17, and qki exhibited lower mRNA expression in the colons of the knockout mice, and ingested BRBs maintained their expressions at normal levels (Fig. 3). In addition, BRBs maintained DNA methylation homeostasis of several Wnt pathway negative regulatory genes in the knockout mice (Fig. 4). Moreover, we found that expression of proteins involved in DNA methylation was decreased by BRBs in the knockout mice (Fig. 5). Thus, our data imply that one mechanism whereby BRBs inhibit colonic ulceration...
occurs by preventing epigenetic dysregulation of the Wnt signaling pathway, in part by deterring DNA hypermethylation, inappropriate histone modifications, and condensation of chromatin. Further research is required to ascertain whether BRBs actually inhibit methylation or rapidly remove methyl groups. The results for *dkk2* and *dkk3* were different. The expression of *dkk2* mRNA in colon tissues of knockout mice fed
control diet was not significantly different from colon collected from WT mice fed either control or BRB diet (Fig. 3D). However, dkk2 was relatively hypermethylated in the knockout mice fed control diet, and BRBs decreased the DNA methylation of dkk2 in the knockout mice (Fig. 4D). In contrast, transcription of dkk2 in knockout mice that consumed BRBs was significantly upregulated to levels that were greater than noted for the control mice (Fig. 3D). Thus, within an inflammatory environment, BRBs both antagonized the hypermethylation of this gene while augmenting its expression. As noted for dkk2, BRBs specifically stimulated expression of dkk3 in colon collected from the knockout mice. These results suggest a novel mechanism that makes BRBs effective in stimulating expression of Wnt signaling negative regulatory genes that remains to be elucidated.

IL-10 is known to regulate growth of commensal flora in the gut and, thus, it also maintains gut homeostasis (32). We have evidence that dietary BRBs increase the abundance of Lactobacillus, Coralobacteria, Bifidobacteriales, Bacteroides, and Clostridiales in F344 rats (unpublished data). The Bray–Curtis analysis showed a strong time-dependent change in bacterial diversity of the microbiota in response to dietary treatment with BRBs (unpublished data). It is possible that BRBs may also have effects on the microbial flora of WT and knockout mice. We speculate that if BRBs exhibit effects on the flora, this could potentially lead to less inflammation as witnessed in the knockout mice. We are currently evaluating the effects of dietary BRBs on gut flora in WT and the knockout mice.

The IL-10 knockout mouse model is a genetic animal model that recapitulates the genesis of human colon cancer that originates within an ulcerative colitis lesion (14). Our current study shows that proper governance of the Wnt signaling pathway is maintained by BRBs by sustaining correct methylation patterns and expression of Wnt pathway negative-regulatory genes. Cancer chemoprevention by affecting histone modification and/or DNA methylation and DNMT1, DNMT3b, HDAC1, HDAC2, and MB2 is a common observation. Indeed, Schnekenburger and Die- derich (9) recently listed 52 dietary foods and constituents that exhibit these activities. We have now shown that ingestion of BRBs prevent the onset of their aberrant actions in the knockout mice. One question that remains, however, is whether BRBs prevent upregulation of DNMT1, DNMT3b, HDAC1, HDAC2, and MB2, or cause their downregulation, or both. Another question is whether they affect expression of these enzymes/proteins directly or indirectly. Further research is ongoing to clarify these questions.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L.-S. Wang, T.H.M. Huang, J. Yu Development of methodology: J. Yu, Y.-W. Huang Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-T. Kuo Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): L.-S. Wang, M. Yearsley, K. Oshima, C.-D. Stoner, J.F. Lechner, Y.-W. Huang Writing, review, and/or revision of the manuscript: L.-S. Wang, G.D. Stoner, J.F. Lechner Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-T. Kuo, Y.-W. Huang Study supervision: L.-S. Wang

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