Multi-omics Approach Reveals Distinct Differences in Left- and Right-Sided Colon Cancer

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Abstract

Increasing evidence suggests that left-sided colon cancer (LCC) and right-sided colon cancer (RCC) are emerging as two different colorectal cancer types with distinct clinical characteristics. However, the discrepancy in the underlying molecular event between these two types of cancer has not been thoroughly elucidated to date and warrants comprehensive investigation. To this end, an integrated dataset from The Cancer Genome Atlas was used to compare and contrast LCC and RCC, covering mutation, DNA methylation, gene expression, and miRNA. Briefly, the signaling pathway cross-talk is more prevalent in RCC than LCC, such as RCC-specific PI3K pathway, which often exhibits cross-talk with the RAS and P53 pathways. Meanwhile, methylation signatures revealed that RCC was hypermethylated relative to LCC. In addition, differentially expressed genes (n = 253) and differentially expressed miRNAs (n = 16) were determined between LCC and RCC. Especially for Prostate Cancer Susceptibility Candidate 1 (PRAC1), a gene that was closely associated with hypermethylation, was the top significantly downregulated gene in RCC. Multi-omics comparison of LCC and RCC suggests that there are more aggressive markers in RCC and that tumor heterogeneity occurs within the location-based subtypes of colon cancer. These results clarify the debate regarding the conflicting prognosis between LCC and RCC, as proposed by different studies.

Implications: The underlying molecular features present in LCC and RCC identified in this study are beneficial for adopting reasonable therapeutic approaches to prolong overall survival and progression-free survival in colorectal cancer patients. Mol Cancer Res; 1–10. ©2017 AACR.

Introduction

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females (1). Typically, the cecum, ascending colon, hepatic flexure, and transverse colon are classified as the right colon, and the descending colon and sigmoid colon are classified as the left colon (2). It is known that the right and left colons have different embryologic origins (right colon arises from the embryonic midgut, and left colon originates from the hindgut), microenvironments, and distinct blood supplies (3). In addition, left-sided colon cancer (LCC) and right-sided colon cancer (RCC) showed distinct differences in epidemiology, biology, pathology, genetic mutations, and clinical outcomes (3–5).

It has been shown that RCC patients were older, had increased tumor sizes and more advanced tumor stages, were more often female, and had poorly differentiated tumors (5, 6). In addition, increasing numbers of studies demonstrated a poor survival in RCC compared with LCC (7). Recently, Petrelli and colleagues (8) found that LCC was associated with a significantly reduced risk of death, which was independent of stage, race, adjuvant chemotherapy, year of study, number of participants, and quality of the included studies through interrogating 1,437,846 patients from publicly available data. However, the underlying molecular events associated with huge difference between LCC and RCC are poorly understood. In this study, we comprehensively characterize the somatic mutations, genome-wide transcriptional (mRNA and miRNA), and epigenetic (DNA methylation) profiles of the LCC and RCC combined with the correlative analyses of the expression, methylation, and clinical data from the Cancer Genome Atlas (TCGA).

Materials and Methods

Mutation data retrieval and processing

The colon adenocarcinoma (COAD) somatic mutation data and clinical information were downloaded from the TCGA data portal (March 2, 2015). Silent mutation and RNA mutation
were discarded. Next, clinical information (i.e., neoplasm location) of each patient was added right after the mutational genes via the unique patient ID. Frequent mutational gene sets (FMGS) with point mutations and small insertions/deletions and hidden association rules (AR) were mined as in our previous work (9). Samples with ambiguous location annotation were excluded.

HM450k data retrieval and process

COAD level three DNA methylation data (HumanMethylation450) were downloaded from the TCGA data portal (March 10, 2015). The methylation level of each probe was measured as the beta value ranging from 0 to 1, which is calculated as the ratio of the methylated signal to the sum of the methylated and unmethylated signal. Probes with an ‘NA’ value in more than 10% of the LCC or RCC samples were discarded. Next, bunphunter Bioconductor package was used to seek the differentially methylated region (DMR) in the remaining probes (10). GRCh37 genome annotation was retrieved from Ensembl (http://grch37.ensembl.org/index.html) since the TCGA level three DNA methylation data (HumanMethylation450) using GRCh37 for probe annotation. The circular diagram was plotted by the RCircos package (11).

Gene expression data processing and normalization

All level three mRNA expression datasets (RNASeqV2) were obtained from the TCGA (October 2015). Gene expression data analysis was performed similar to our previous work (12). Briefly, differentially expressed mRNA analysis between LCC and RCC was performed by the limma package for R/Bioconductor. Genes with an expression level < 1 (RSEM-normalized counts) in more than 50% of the samples were removed. Significantly differentially expressed mRNAs were selected according to the FDR-adjusted P value < 0.05 and fold change > 2 condition. Validation mRNA expression dataset was downloaded from the Gene Expression Omnibus database under the accession numbers: GSE14333. The dataset was explored similarly to what was mentioned previously. Volcano plots were created with the ggplot2 package (http://ggplot2.org/).

Integrated analysis of miRNAs and mRNAs

Differentially expressed miRNAs between LCC and RCC were explored similar to differentially expressed genes (DEG). Putative target genes of differentially expressed miRNAs were predicted using miRanda (13) and TargetScan (14). Intersection of putative target genes and DEGs was retained for further analysis. We calculated correlation between miRNA and its mRNA targets and retained negatively correlated pairs (Pearson correlation coefficients < 0 and P < 0.01). MiRNA–mRNA interaction network was constructed by using Cytoscape (v3.2.1).

Network construction

Matched mRNA–miRNA samples were retained for coexpression analysis. The coexpression network was constructed by weighted correlation network analysis (WGCNA) package for R (15) and explored as in our previous work (12). The subnetworks constituted by the top 50 hub genes in the specific module were visualized by VisANT (16). Next, the top 50 hub genes were subjected to Gene Ontology (GO) interrogation. The P value was determined by the hypergeometric test with the whole annotation as reference set and then adjusted for multiple testing using the Benjamini–Hochberg FDR correction method. GO enrichment analysis was conducted by BINGO implemented in Cytoscape (17, 18). All statistical analyses and graphical representations were performed in the R programming language (× 64, version 3.0.2) unless otherwise specified.

Immunohistochemistry staining

Fifteen LCC and 32 RCC formalin-fixed paraffin-embedded (FFPE) samples were collected from the Second Affiliated Hospital, Zhejiang University School of Medicine between 2010 and 2016. Immunohistochemistry (IHC) staining was performed similar to our previous work (19) except for antibody was replaced with anti-PRAC (1:500 dilution, Invitrogen, PA1-46237).

Results

Sparse somatic mutation pattern in RCC

Here, we used the Apriori algorithm to find the FMGSs with point mutations and small insertions/deletions from 84 LCCs and 145 RCCs as part of the TCGA Pan-Cancer effort. Totally, 65 and 326 unique k-1 FMGSs were identified in LCC and RCC, respectively. The largest FMGS size identified in LCC and RCC was three. Deeper analysis of the FMGSs showed that 8-fold more one-itemsets (k = 1) of FMGSs were found in RCC than LCC (Supplementary Table S1). Though the top driver mutation genes were basically the same, the pattern of the mutation frequencies differed greatly. In LCC, APC (support: 0.835) and TP53 (support: 0.647) were absolutely the dominant driver mutation genes. However, in RCC, a decreasing gene mutation pattern was observed. Despite that finding, APC and TP53 remained the top mutation genes. The sparse mutation pattern in RCC results in more co-occurred driver mutation genes. Except for TP53 and APC, other drivers (e.g., RNF43, FAT4, PIK3CA) also frequently co-occurred with other cancer-related genes in RCC. For example, in RCC, RNF43 often co-occurred with, for instance, SYNE1, OBSCN, BRAF, and LAMA5 (Supplementary Table S1).

It is well-known that microsatellite instability (MSI) patients have an elevated frequency of single-nucleotide variants because of inactivation of the DNA mismatch repair (MMR) system (20). MSI is observed in 15% of sporadic colorectal tumors diagnosed in the United States (21, 22). Therefore, we further divided the LCC and RCC into MSI-high (MSI-H) and microsatellite stable (MSS) subgroups according to the clinical annotation by TCGA (23) and the findings by Hause and colleagues (21). Intriguingly, we found that the proportion of MSI-H in RCC was 20% (29 samples) and only approximately 5% in LCC (4 samples). In this context, we speculated that the sparse somatic mutation pattern in RCC may be biased by the MSI-H samples since the MSI-H samples had an elevated mutational load. As such, we independently calculated the FMGSs in MSS and MSI-H subgroups of RCC. However, similar results held in the MSS of RCC, with 127 unique k-1 FMGSs and 68 one-itemset (k = 1) FMGSs, which is 2-fold and 3-fold more than in LCC, respectively.

Regarding the RCC-MSI-H, a much higher mutational burden was observed, with 1,026 mutated genes being observed per
Tumor. In addition, as many as 200 one-itemset ($k = 1$) FMGSs with support > 0.3 were found in RCC-MSI-H. Especially for RNF43, the frequent and validated mutation target in MSI-H cancers (21, 24), was the most significantly mutated gene in RCC-MSI-H (support: 0.76). Nevertheless, an exclusivity of the MSI-H status and canonical colorectal cancer driver mutation genes, such as APC (support: 0.24 vs. 0.73 in MSS), TP53 (support: 0.24 vs. 0.53 in MSS), and KRAS (support: 0.28 vs. 0.45 in MSS), were observed. This finding may account for a better MSI-H patient survival than MSS patients in colorectal cancer. Collectively, our data revealed that LCC and RCC had distinct driver patterns that should be considered in future targeted cancer therapy.

Hypermethylation in RCC

Changes in the DNA methylation status are thought to be involved in colorectal cancer initiation and development. Therefore, we attempted to identify the DMRs to decipher the epigenetic difference between LCC and RCC. Notably, 552 DMRs (bumps size ≥ 2, cutoff = 0.2, permutation test $P < 0.01$, Supplementary Table S2) were determined using bumphunter. Among them, 499 DMRs (92%) were located within CpG island, and 27 DMRs (5%) were located outside CpG island.

Figure 1.
Identification of the DMRs between LCC and RCC. Notably, 540 hypermethylated regions were found in RCC, and only 12 hypermethylated regions were found in LCC. The innermost circle corresponds to the RCC methylation signature (depicted by red scatter plots), and the outer circle corresponds to the LCC methylation signature (depicted by blue scatter plots). The scatter location in each track is corresponding to the methylation level (0–1). Selected DMR-related genes are present inside the hg19 human chromosome ideogram. An example of RCC hypermethylated regions relative to LCC were shown in the center of the circle.
were in the shores. Notably, a hypermethylation profile (540 DMRs) was observed in RCC (Fig. 1). Of the 12 hypermethylated DMRs (34 probes) in LCC, 3 DMRs (13 probes) were associated with HOX family members HOXB5, HOXB7, and CDX2. GO enrichment analysis showed that the DMR-associated genes were mainly enriched in organ morphogenesis (Benjamini–Hochberg FDR correction 3.1798E–10, 42 genes), cell differentiation (6.8791E–9, 71 genes), and embryonic morphogenesis (2.0480E–9, 29 genes).

Activation of onco genes and silencing of tumor-suppressor genes in RCC

Using mRNAseq data compiled in TCGA, LCC versus RCC revealed 253 DEGs (fold change > 2 and adjusted P value < 0.05) between them. In LCC, 105 genes were upregulated, and 148 genes were downregulated, compared with the RCC (Fig. 2A). Functional annotation of these DEGs uncovered that oncogenes (e.g., ZIC5) were overexpressed in RCC, whereas tumor-suppressor genes (e.g., HOXB13) were repressed. To further confirm the DEGs identified using TCGA data, the GSE14333 dataset with 122 LCCs and 126 RCCs was used for identifying DEGs. In total, 330 DEGs were identified (Fig. 2B), with 36 of them overlapping with the TCGA result (Fig. 2C). The Prostate Cancer Susceptibility Candidate (PRAC) was the most significantly downregulated gene in RCC (Fig. 2D and E). To validate this result, 15 LCC and 32 RCC FFPE samples were used for IHC staining. Results showed that both the IHC staining score and positive rate in LCC were significantly higher than in RCCs (Supplementary Fig. S1). The function of PRAC in COAD remains elusive; however, another highly downregulated gene in RCC, HOXB13, approximately 2 kb downstream of PRAC, has been shown to be a colorectal cancer suppressor (25, 26). And HOXC6, which also belongs to homeobox transcription factor (TF) family, was the most significantly upregulated gene in RCC (Fig. 2F and G).

Further correlation analysis of the DNA methylation and expression showed that 13 downregulated genes (CLSTN2, ECEL1, ELAVL2, GDPF10, LMX1A, PRAC, PRKAA2, RSPO4, SNX32, ST6GAL2, TAC1, VENTX, and WASF3) in RCC were possibly repressed by hypermethylation of DMR. For example, one DMR, situated in the CpG island shore, was strongly associated with PRAC repression in RCC. The hypermethylated DMR in RCC encompassed 8 probes (cg00960395, cg04574034, cg09866983, cg12374721, cg14230397, cg20094830, cg20945566, and cg27170782) that cover 388 bps in chromosome 17 (Fig. 3). DNA methylation has been shown to play a role in the regulation of PRAC expression in prostate cancer (27).

Figure 2.
Identification of the DEGs between LCC and RCC. A, Volcano plot revealed that 135 genes were overexpressed in RCC, and 118 genes were underexpressed in RCC using TCGA data. B, Volcano plot uncovered 97 genes were overexpressed in RCC, and 233 genes were underexpressed in RCC using publicly available GSE14333 data. C, Venn diagram revealed that 36 DEGs were overlapped between TCGA and GSE14333. For D to G, boxplot distribution of PRAC and HOXC6 expression level by using TCGA (D and F) and GSE14333 (E and G) data.
miRNA expression

In addition to mRNA, we also want to know if expression discrepancies exist between LCC and RCC in noncoding RNAs. Interestingly, 15 cancer-related miRNAs were differentially expressed (adjusted P value < 0.05 and fold change > 1.5) between LCC and RCC. Eight miRNAs, namely, miR-196b, miR-466, miR-296, miR-552, miR-1247, miR-1275, and miR-3131, were downregulated in RCC, whereas seven miRNAs were upregulated, including miR-10b, miR-31, miR-146a, miR-155, miR-615, miR-625, and miR-1293 (Fig. 4A). The abundantly expressed miR-10b, an miRNA closely associated with cancer metastasis, had significantly higher expression level in RCC (Fig. 4B).

Further integrated analysis of miRNA and DEGs showed that 16 DEGs were putatively regulated by seven miRNAs (Fig. 4C). Two miRNAs, miR-625 and miR-146a, were highly expressed in RCC, and their predicted targets were all downregulated in RCC. As for the other low expression of miRNAs in RCC, their predicted targets were upregulated in RCC. For example, miR-466 was downregulated, and its predicted target, ZIC5, a transcriptional repressor that elevated expression contributed to cancer progression, was upregulated in RCC.

RCCs tend to be more aggressive through orchestrating invasive gene modules

Considering the huge expression difference between LCC and RCC, we speculate that the regulatory network also differs greatly. To this end, we used WGCNA to explore the core gene regulatory modules, which may shed light on the intrinsic expression variation between LCC and RCC. Interestingly, six and nine modules consisting of at least 100 genes were found in LCC and RCC, respectively. Five modules related with mRNA metabolic process, DNA ligation, regulation of ARF GTPase activity, translational elongation, vasculature development, cell adhesion, complement activation, T-cell activation, and lymphocyte activation were shared by LCC and RCC (Fig. 5, linked by colored band). Of note, because certain modules such as brown- and blue modules in LCC were rather large. Therefore, the top 50 hub genes were extracted in each module. We found that no miRNAs ranked in the top hubs in the module. This observation raises the possibility that miRNA, unlike TF, has a restricted role or at least does not act as a key orchestrator in COAD. The module peculiar to LCC was linked to cellular nitrogen compound metabolic process (FDR-corrected P value 3.3739E–2, 13 genes). The rest of the four RCC-relevant modules were linked with cell-cycle phase (magenta module, 5.4696E–28, 27 genes), mitosis (magenta module, 2.9294E–29, 24 genes), generation of precursor metabolites and energy (brown module, 1.0886E–4, nine genes), microtubule-based process (purple module, 3.1047E–2, five genes), response to virus (7.5314E–8, nine genes), and innate immune response (salmon module, 4.4101E–2, four genes). As such, enhanced activation of cell division, energy metabolism, and immune system process in RCC underscores the speculation of its more aggressive state.

Pathway alteration difference between LCC and RCC

Integrated comparison of mutations and mRNA expression alteration in 85 LCCs and 116 RCC-MSS will enable better understanding of the intrinsic difference in the pathway dysregulation. Alteration frequency between LCC and RCC-MSS was examined in five well-known pathways (WNT, RAS, PI3K, TGF-beta, and P53) that are closely associated with colorectal cancer. Basically, the mutation rate in RCC-MSS is much more frequent than LCC in TGF-beta, PI3K, and RAS signaling pathways, but almost the same in WNT and TP53 pathways (Fig. 6). The WNT signaling pathway was altered in 89% LCC and 85% RCC-MSS, respectively, which mainly contributed by inactivation of APC (78% and 73%), followed by the mutation of FAM123B, FBXW7, SOX9, CTNNB1, AXIN2, and LRPIB. Downregulation of LRPIB in colon cancer has recently been shown to be associated with promoting growth and migration of cancer cells (28). In the TGF-beta pathway, higher mutation rates (LCC vs. RCC-MSS, SMAD2, 0% vs. 3%; SMAD3, 1% vs. 4%; SMAD4, 12% vs. 16%) in RCC-MSS were observed in SMAD family members. As for the PI3K pathway, PIK3CA was mutated in 11% LCC samples and 26% RCC-MSS samples. The case in PI3K, a much higher mutation rate of KRAS (30% vs. 45%) and BRAF (7% vs. 11%) was found in RCC-MSS (Fig. 6). Activation of the PI3K and RAS pathways may be more prone to progression for RCC-MSS.

To further explore the co-occurrence of the dysregulation of different pathways in LCC and RCC-MSS, we re-examined the FMGSs that were identified as mentioned earlier. The results showed that five FMGSs (k > 1, support > 0.1) were in common...
between LCC and RCC-MSS. Inactivation of WNT and P53, but activation of the RAS pathway, occurred simultaneously in more than 15% of the COAD samples (both LCC and RCC-MSS). However, we observed nine more examples of RCC-MSS–specific pathway cross-talk that have >10% support (Fig. 6, right bottom plot). In addition to the interaction with the WNT pathway, the PI3K pathway often crosstalks with the RAS and P53 pathways in RCC-MSS. In addition, in RCC-MSS, the TGF-beta pathway also often interacted with the RAS and WNT pathways via SMAD4 and KRAS or APC, respectively. Of note, we also observed single pathway members that were inactivated simultaneously, represented by APC and SOX9 (support = 0.121) or FBXW7 (support = 0.103) in the WNT pathway. Collectively, we found numerous molecular signatures that were associated with tumor aggressiveness in RCC-MSS, which suggests a poor prognosis for RCC-MSS patients.

**Discussion**

The large molecular difference between LCC and RCC identified in this study consolidates the different embryonic origins of LCC and RCC. And the difference is independent of sex ($X^2$ test, $P = 1$). Actually, DNA methylation alteration is an early event that occurs in cancer. Koestler and colleagues (29) determined that 168 probes enriched in homeobox genes that were differentially methylated between the right- and left-colon adenomas using the Illumina HumanMethylation450 BeadChip. Consistent with their observation of PRAC hypermethylation in right-colon adenomas and hypermethylation of CDX2 in left-colon adenomas, the difference also held true in the carcinoma.

As a TF, HOXC6 promotes prostate cancer metastasis via inducing aberrant metastatic miRNA expression (30). It
is tempting to believe that this result held in RCC due to the elevated expression of HOXC6. Previous studies demonstrate that the overexpression mir-10b can promote tumor invasion (31, 32) and confers chemoresistance in colorectal cancer cells to 5-fluorouracil (33). In this context, 5-fluorouracil may not be suitable for the treatment of RCC due to a much higher expression level of mir-10b in RCC than LCC. In addition, high mir-10b expression in primary colorectal cancer tissue can independently predict distant metastasis (34), which suggests that primary RCC is more vulnerable to metastasis. Other oncomiRNAs (mir-31, miR-155, miR-625) that were overexpressed in RCC were all closely associated with colorectal carcinoma cell proliferation, migration, and invasion (35–37). In contrast, two tumor-suppressor miRNAs in colorectal cancer, miR-296 and miR-592, were underexpressed in RCC. MiR-296 inhibits the metastasis and epithelial–mesenchymal transition by targeting S100A4 and miR-592, which inhibits cell proliferation by suppressing of CCND3 expression (38, 39).

It must be noted that a more aggressive status was observed in RCC through determining the modules consisting of tightly coregulated genes. The large brown module unique to RCC harbors 1,353 genes that were mainly involved in electron transport chain. In addition, three other RCC-specific modules were enriched in cell-cycle regulating, mitosis/nuclear division, microtubule-based process, and innate immune response, which were all linked to tumor aggressiveness. In parallel, as described earlier, miRNAs tend not to be hub genes with an extremely high connectivity. This finding can be ascribed to the intrinsic target recognition discrepancy between plant and animal miRNAs (40). In plants, almost all miRNA targets have a single miRNA-responsive element and are regulated by just one miRNA or one miRNA family through nearly perfectly complementary miRNA-target pairs. Thus, the scope of miRNA targets appears to be only a handful of key targets, most of which are TFs. In animals, miRNAs target transcripts via imperfect base-pairing to multiple sites in the 3′ untranslated regions by the seed sequence (5′ end of miRNAs that comprises nucleotides 2–7). For this reason, over 90% transcripts were putatively targeted by miRNAs, and one transcript was often under combinatorial control of multiple miRNAs (41, 42). In this context, animal miRNAs generally act as fine-tuners that exert wide subtle regulatory effects across the transcriptome and tend not to be hub genes in the regulatory network.

PRAC, a small protein with a molecular weight of only 6 kDa, has been shown to be specifically expressed in the human prostate, rectum, and colon (27, 43). In prostate cancer, the expression level of PRAC is significantly downregulated compared with benign prostatic hyperplasia. Here, we demonstrated that its expression level is significantly downregulated in RCC, which is in line with the findings by Bauer and colleagues (44). Although the concrete function of PRAC remains elusive in COAD, a possible cotranscribed gene HOXB13 has been shown to be involved in suppressing colorectal cancer progression (25, 45). In RCC, HOXB13 was significantly downregulated, as well. Thus, PRAC may serve as a tumor suppressor through interacting with HOXB13 in COAD.

In addition, RCC-MSS had more mutated BRAF (11% vs. 7%) and KRAS (45% vs. 30%), and similar results were observed in our Zhejiang University Cancer Institute–collected samples through sequencing 338 pairs of matched fresh frozen colorectal cancer tissue and adjacent normal tissue samples (unpublished data). BRAF mutation has been shown to be associated with a worse prognosis (46) and a poorer response to cetuximab therapy (3, 47). In addition, colon cancer patients with wild-type KRAS treated with cetuximab have significantly improved overall survival (OS; ref. 47). Thus, a worse prognosis in RCC can be partly ascribed to a higher mutation rate of KRAS.
and BRAF (Fig. 6). More recently, Ptashkin and colleagues (48) find that chromosome 20q amplification is associated with a better OS in LCC. In addition, because cetuximab is mainly used in advanced stages of colorectal cancer patients, FMGS mining is further conducted in four pathologic stages according to the American Joint Committee on Cancer standards. Notably, we found KRAS harbored great stage heterogeneity between LCC (stage I: 25%, II: 53%, III: 9%, and IV: 20%) and RCC (53%, 46%, 31%, and 62%). Nevertheless, we should bear in mind that current targeted drugs used in treating colon cancer patients are rather limited and that the prognosis may be reversed if the targeted drug is based on mutated KRAS or BRAF. Meanwhile, OS and progression-free survival (PFS) in RCC can benefit from combination therapy since frequent pathway cross-talk occurred in RCC.

Recently, Le and colleagues (49) demonstrated that PD-1 blockade works well in MMR-deficient colorectal cancer, and prolonged PFS was associated with high somatic mutational loads. In this manner, RCC patients that are deficient in MMR, approximately 20% of RCC, can benefit from PD1 therapy (23, 50). In short, the underlying molecular feature present in LCC and RCC identified in this study is beneficial for medical decision-making and prolonged OS and PFS. Our results help to elucidate the molecular basis of treating LCC and RCC as two different diseases in future cancer treatment.

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

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Conception and design: W. Hu, W. Ge, S. Zhang, S. Zheng
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