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Title:

Extensive genomic plasticity identified by anti-cancer Huaier therapy

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Transcriptional control

Clinical significance of anti-cancer effects of Huaier has been emphasized recently. We have proved that a broad spectrum of Huaier effects was based on the rescue of the disrupted Hippo signalling pathway, especially through the rescue of transcriptional dysregulation. We initiated clinical research for thorough understanding of mode of action by total RNA- and small non-coding RNA-sequencing. Here we provide the surprising genomic plasticity by Huaier observed in the time course of recovery from cancer. The extensive changes in RNA editing events were first observed, such as average 92,427 SNP variances per individual (22,688 in normal control). The subsequent changes in the process of translation and transcription resulted in the drastic changes in number of up/down-regulated transcripts. The ratio of the changes at maximum was 85% (23,210/27,447). With the advent of many novel sequences found in small nuclear RNAs and the consequent gene-silencing derived from miRNA-mediated post-transcriptional control, quantitative and qualitative changes of expression in transcriptional factors (mean 1,115/person) contributed to rescue dysregulated functions occurred in almost all required physiological functions for cancer recovery. The transcription control was demonstrated as functional lineage map between the core transcriptional factor and functionally linked transcripts, and the recovery could be clearly shown as the silencing of those massive changes in the end. The genetic alterations, especially related to the inhibition of NF κ B and TGF- β signalling pathways showed significant effects on the improvement of prognosis. Thus, Huaier therapy contributes to cancer cell death with damaged tissue regeneration by extensive and systematic genomic modulations.

Recent advances in cancer therapy integrate most advanced devices and inventions among broad areas of science¹⁻³. Although a rapid progress in the methodology⁴⁻⁸, many tasks remain unsolved for successful control of cancer, and for diseases originated from multiple factors and environmental stresses throughout a life-long time⁹. Recent advancement in technology enabled to cultivate those useful natural herb species in quantity¹⁰⁻¹⁴, and quality-controlled distribution in the conventional granule forms. In 1970's, Huaier (*Tremetes robinophila* Murr) has been recognized widely as anti-cancer drug in China¹²⁻¹⁴ (Chinese administration license No. Z-20000109).

Huaier has been introduced with its significant anti-cancer effects without any side effects and/or toxicity¹³⁻²³. The effective ingredient of Huaier was proteoglycan, which consists of 41.53% polysaccharides, 12.93% amino acids and 8.72% water^{13,14}. The proteoglycan is the most effective anticancer element among all of the isolated ingredients of Huaier extract, which was confirmed on breast cancer MCF-7¹⁵, liver cancer H22^{16, 17}, MHCC97-H¹⁸, lung cancer¹⁹, ovarian cancer²⁰, colorectal cancer²¹, and human fibrosarcoma HT1080 cells²². More importantly, Huaier has no toxicity or side provided a simple and successful evidence that the rescue of Hippo signalling pathway was a molecular basis of anti-cancer effects of Huaier^{4, 5}. However, it was not enough, either, to explain all functions associated to anti-cancer effects, such as 1) immunological alterations both to enhance and also inhibit the excessive reaction, 2) normal tissue reproduction after the specific cell death and elimination of cell debris in cancer lesion, and 3) reduction of (oxidative) stress materials in liver, and 4) recovery from the opportunistic infections during cancer therapy, and etc.

Therefore, we initiated clinical research to identify Huaier effects on every biophysiological system and influences each other. The samples used in this approach should be free from the effects of chemotherapeutic agents, especially molecular-targeting anti-cancer drugs, which disrupted largely molecular mechanisms. The present study was thus constructed with a collaboration of cancer patients, not a large in number, but wished to have Huaier treatment.

The medical characteristics of the 31 volunteer patients were summarized in **Table 1**. The mean age is 67 years old, since this research excluded the congenital cancer or tumour. The present study summarizes the results from 92 sample sequencing from 31 patients, including 2 normal controls (patient No. 21 and 26), and 4 with benign tumours (No. 13, 14, 18, and 20). Normal control No. 21 was the same as No. 25, the opportunistic infection case after 8 months of the end of first research period as No. 21. In order to compare the results with the other volunteer patients with opportunistic virus infections, the sampling was continued over one year from the patients No.5, 8, and 10. The sample from the volunteer No. 20 without Huaier administration was later identified adenoma progression in colon, and successfully removed by colon endoscopic dissection one month later.

The cancer origin and the stages (benign tumour to cancer TNM stages 0 to IV) varies among patients, and their symptom changes and anti-cancer effects after Huaier administration were confirmed by many ordinary clinical tests in clinics, and by the evaluation of CT, MRI, and X-ray images. The volunteers summarized in **Table 1** were free from any conventional anti-cancer chemotherapy during research period (3 months). TNM Stage IV patients were only treated by Huaier.

Interestingly, there were 5 volunteers who had virus infection during the observation period (No. 5, 8, 10, 12, 21/25). The methodology hired in the present study was also valid to identify virus infection, and that could analyse in quantity (**Fig. 4b**). These results confirmed that Huaier could not prevent virus infection, however, the infection remained silent and totally cured within reasonable period (one month to half a year) according to the severity of the condition of original disease.

The obtained peripheral blood samples (92 samples from 32 cancer patients) were designated to be analysed by RNA extraction, construction of whole transcriptome library for sequencing on BGISEQ-500 Platform, followed by whole transcriptome mapping^{3,7,8,25-28}. We have also performed total non-coding small RNA sequencing on the same BGISEQ-500 Platform^{3,7,8, 25-28}, and the length of the small RNAs were found to be between 18 to 30 nucleotides.

The results were compared between the samples obtained before and after 1 month, and 3 months of Huaier administration. The quantitative analysis of > 7.0 GB RNA sequencing of each sample provided estimated transcribed genes of >27,000; mean number of 27,447 per sample (**Table 2**). The average mapping ratio with reference genome is 89.64 % (varies from 71.49% to 94.51%); 43,296 genes were identified per sample (in which 18,952 of them are already known genes). and 24,344 of them are novel genes. Total number of the novel transcripts identified was 172,487, composed of 1) 108,991 for previously unknown splicing event for known genes, 2) 24,344 novel coding transcript without any known features, and 3) 39,152 long non-coding RNA. The obtained novel genes and small nuclear non-coding RNA obtained in the present study have been deposited to the NCBI GEO (GSE157086).

The sequencing reads were filtered to remove low-quality, adaptor-polluted and high content of unknown base (N) reads. After reads filtering, we mapped those clean reads only to reference genome using HISAT1²⁹, and confirmed the sample quality. At the same time, the bam files for genome mapping results were prepared using Integrative Genome Viewer (IGV)³⁰ provided by BGI. This process supported importing multiple samples for comparison and show the distribution of reads in the exon, intron, UTR, intergenic areas based on the annotation results.

After genome mapping, STRINGTIE³¹ was used to reconstruct transcripts, and with genome annotation information, novel transcripts were identified by using Cuffcompare (a tool of Cufflinks)³², and the coding ability of those new transcripts were predicted by CPC (Coding Potential Calculator)³³.

At the same time, we used GATK (Genome Analysis Toolkit)³⁴ to identify single nucleotide polymorphisms (SNP) and insertions and deletions (INDL) variants for each sample. Final results are shown in **Fig. 1a**. We identified total 8,503,297 SNP variant types in 92 sample analysis, and 92,427 SNP per sample in mean number (ranging from 48,994 to 146,102), whereas 22,688 in total among normal healthy individuals³. As for the SNP variations, A-G > C-T transitions were the most common mutations (3,152,487 and 3,142,470 respectively, 74.0%), followed by A-C > A-T (552,611 and 406,169, respectively, 11.3%), C-G > G-T (700,403 and 549,157 respectively, 14.7%) transversions, which is consistent with the previous reports on oesophageal squamous carcinoma cells⁸. Mean number of transitions per sample is 68,423 (ranging from 34,672 to 110,293), whereas 24,004 transversions (ranging from 14,322 to 35,809) (**Table 2**).

These ratios found no significant differences among samples, but such a gross increase of SNP variants has not been reported before using the samples from the same person. The incidence ratio was three times higher in transition, which is much higher than that identified in oesophageal squamous carcinoma cells⁸, breast cancer cells¹⁶, and other cancer cells¹⁷⁻²¹ analysed before. There were no significant differences identified by cancer origin, gender, and the stages of cancer. In contrast, the significant increases were observed at the time of opportunistic virus infections in patient No. 8, 10, 12, and 21.

Distribution of SNP location is about 40% each in both exons and introns, and the rest located in intergenic region (12%) and within 2,000 bp up- and down-stream region (2% each) of the expressed genes. The distribution of numerous INDL (>2,000,000 per sample) location showed the similar results. Both SNP and INDL location was identified 40 to 45 % in exons, and up to 60% including the adjacent area. Thus, These RNA editing events did not randomly scatter among the whole genome, nor any correlation to cancer origin or stages.

We also detect the level of differentially splicing genes (DSG) compared before and after Huaier administration, using rMATS³⁵. The summary of splicing variation comparison is shown in **Fig. 1b**. DSGs are regulated by alternative splicing (AS), which allows the production of a variety of different isoforms from one gene only. Changes in relative abundance of isoforms, regardless to the expression change, indicates a splicing-related mechanism. We detected five types of AS events, including skipped exon (SE), alternative 5' splicing site (A5SS), alternative 3' splicing site (A3SS), mutually exclusive exons (MXE), and retained intron (RI). Approximately total 2,557,753 splicing events in 92 samples were observed, in which were 1,303,783 SE (51%); 211,336 MXE (8.2%);

264,368 A5SS (10.3%); 343,124 A3SS (13.4%); and 435,142 RI (17.1%), respectively. Mean numbers of each event per sample (= per person) were 14,172 (ranging from 7,567 to 19,240) SE; 2,297 (ranging from 1,229 to 3,258) MXE; 2,874 (ranging from 1,655 to 3,704) A5SS; 3,730 (ranging from 2,341 to 4,699) A3SS; and 27,802 (ranging from 16,294 to 36,079) RI, respectively. Just like SNP variants, the ratio of splicing events showed no significant differences among patients, and irrespectively to cancer origin, cancer stage, age, or gender (**Table 2**), only exception at the time of virus infections.

Both results clearly indicated the genomic events were not influenced by cancer origin, stage, and personal differences, but the extrinsic factors such as virus infections. The important findings were total number of changes, quantitative alterations identified, which clearly indicates significant genomic flexibility and capability in each patient. These results of average numbers of RNA editing events such as SNP and INDL showed that Huaier administration induced approximately 4 times higher movability of the genome, ranging 2 times to 7 times higher than those detected as identified among normal population³. The statistics of splicing events expanded the plasticity and flexibility enhanced the genomic capability to manage or cope with the following transcription and translation. The importance of these results will be discussed later, together with the results of transcriptome analysis and the results obtained by small non-coding RNA sequencing.

After the genome mapping and Gene Ontology classification, we defined 24,344 novel transcripts. Then we merge novel coding transcripts with reference ones to get complete reference, and map clean reads using Bowtie³⁶, followed by calculate gene expression level for each sample with RSEM³⁷. The results provided the total numbers of predicted transcripts were 27,447, and the gene mean mapping ratio was 89.64 % (ranging from 71.49% to 94.51%).

Based on the expression information, box plot analysis was completed to show the distribution of the gene expression level of each sample, followed by construction of the density map which revealed the change of gene abundance by the time course of Huaier administration. As a result of those analysis, gene expression level analysis enabled us to compare the levels of Differentially Expression Genes (DEG) among samples of the same patients according to the time course of Huaier administration (**Fig. 2**).

The numbers of up- and down- regulated DEGs were summarized in **Table 3**. We also used MAplot, Volcano plot, Scatter plot, and Heatmap plot to show the distributions of DEGs (details demonstrated at BGI website: <http://www.bgitechsolutions.com/>). As shown in **Table 3**, the average number of up-regulated genes were 2,589 out of the total 27,447 transcripts (ranging from 21 to 5,671), whereas down-regulated genes were 2,273 (ranging from 28 to 4,227).

Fig. 2 demonstrates those changes by individuals. It is obvious that there were high-responders as well as low-responders to Huaier. Generally, TNM stage IV cancer patients were typical low-responder (such as Patient No.7; colorectal cancer with type I diabetes, with a long-term history of chemotherapy combinations before Huaier taking). A sudden increase of the up-regulated genes was a typical first observation, followed by the inversion to the increase of down-regulated genes. The endoscopic dissection of cancer lesion often caused massive increase of up-regulated genes (patients Nos. 9-11, 13, 14, 19), more or less 10 to 25 % of total transcripts, which might reflect the repair function and tissue reproduction by Huaier. The history of chemotherapy and radiotherapy delayed any responses (5, 6, 9, 24, 27, and 30), typically observed in Patient No. 5.

Interestingly, just as observed in RNA editing events, the opportunistic virus infection caused the utmost increase of up-regulated genes, highlighted by asterisks in **Fig. 2**. Huaier administration successfully down-regulated those changes to normal level, within 30 days in normal control, 3 to 6 months in cancer patients (Patient No. 5, 8, 10), with an exception in patient No.12, most severe case of stage IV lung cancer patients with multiple metastasis without any additional medical care or hospitalization.

As reported, anti-cancer effects of Huaier was significant on hepatocellular carcinoma^{14, 16-18, 23}, breast cancer^{15, 23}, and other cancers¹⁹⁻²³. In the present study, significant effects on the recovery from oesophageal cancer (squamous cell cancer and adenocarcinoma) was observed, and that even with multiple metastasis (patient No. 5, 9, 28, and 29). Interestingly, the patient No.29 with lung metastasis was successfully recovered judged by CT images and endoscopic examination, without any significant increase of both up- and down-regulated transcripts. The clinical examinations showed a good coincidence to the results obtained here, with a significant improvement/elimination of metastatic lesion, and that without any more progression of the disease.

In addition, it is emphasized here that genomic plasticity has been continuously observed even in normal healthy individuals. It seems that, without any medical problems and disorders, every person should have influences from environmental stress, infective agents, ageing processes which disrupted physiological functions. Huaier revealed its efficacy even on the healthy individual, according to the extent of functional disruption. The observation provided of the potential of Huaier to rescue the silent disruptions in molecular level to recover the normal homeostasis.

With quantitative analysis of DEGs, we performed Gene Ontology (GO) classification (**Fig. 3a**) and functional enrichment. Basically, GO has three ontologies: molecular biological function, cellular component and biological process, and the results were compared by each patient with a consideration of time course of Huaier administration. We also performed KEGG pathway classification²⁵ according to each cancer analysed in the present study (<https://www.genome.jp/kegg/>,

Fig.3b) and functional enrichment (**Fig.3c**). Since there were no basic figure on oesophageal cancer in KEGG pathway classification, we did not demonstrate the molecular linkages in this cancer.

There were a broad range of enriched pathway and up- and down-regulations among cases, and the alterations in transcripts were observed among all the molecular function categories, i.e., every process involved in molecular biological function, cellular component and biological process classified by KEGG pathways (the numbers of DEGs were shown in **extended Table 1**). In **Fig. 3**, the typical GO changes were shown by three types of analyse methods. Since abundant numbers and kinds of alterations identified so far, these panels were focused on the influence of Huaier on; a. the opportunistic infection, b. the responsible molecules altered in each cancer and signalling pathway, and, c. the responsible alterations observed in colorectal cancer by progression of the stage (from adenoma to stage IV) by the pathway functional enrichment analysis.

In **Fig. 3a**, the utmost DEG changes were shown. The largest changes were highlighted by red box, the level was 3,477.8 in mean number (ranging from 1,826 to 5,671). These changes were indicated by asterisks in **Fig. 2** and **Table 3**, too. The virus origins in 5 patients, were; HTLV-1 or related RNA viruses (patient No. 5, 10, 12), Papilloma virus (patient No.8), and Epstein-Barr virus (patient No. 21/25) by KEGG classification. Those patients were significantly improved with administration of Huaier, however, the patient No.12 (deceased at 150 days after Huaier administration). Patient No.8 had surgical dissection of facial basal cell cancer just before the last sampling.

As shown in **Fig. 3a**, continuous Huaier administration (20g per day) could completely rescue the molecular functions to the normal level (to recover homeostasis). The required time for the recovery seemed to vary among patients and physiological situation (from 30 days to several months), and severe case such as patient No.12 could not recover from the immunodeficiency treated by Huaier only. However, even in this case, the prognosis was improved up to 150 days, significantly improved with better QOL than predicted.

Immunological improvements seemed to be closely linked to the regulation of NFκB signalling pathways, which also closely related to improve prognosis, as shown in **Fig. 3b** (comparison among patients 8, 21, and 12). **Fig. 3b** demonstrated detailed factors and molecules with up/down regulation according to the KEGG pathway map²⁴ (<https://www.genome.jp/kegg/>). The panels represented Huaier effects on each cancer (noted by upper left) after 90 days administration, with alterations of DEGs indicated by red coloured box (up-regulation) and blue-coloured box (down-regulation). In each panel, the specified DEG names were highlighted in grey box, especially related to oncogenes and tumour suppressor genes. The numbers of DEGs classified by KEGG pathways in all the patients by the time course were shown in **extended Table 1**.

We searched for the possible molecular changes by the comparison between the patients recovered and deceased (patients number noted in **Table 1** were written on the left side of each panel). The representative changes were highlighted in grey box such as: oncogenes; c-Myc, TERT, NRF2, c-Met, PIK3CA, k-Ra, HER2/neu, EGFR, MDM2, PDGF, PDGFR, CDK4, tumour suppressors; Rb, p53, PTEN, p16, Smad4, BRAC2, IKN4a/ARF, AXIN, KEAP1, ARIDIA, and DNA repair genes; hMLH1, hMSH2, hMSH3, hMSH6.

Huaier administration induced as such many clusters of oncogenes and tumour suppressor genes, and that in both up- and down-regulation. It was difficult to identify the specific molecular systems and factors to control cancer recovery by Huaier, and such a broad spectrum of DEG alterations have not yet been reported yet in the same individual. The modification of transcriptomes contained all the molecules currently targeted by anti-cancer chemotherapy. Huaier seemed to compensate for the limitation of targeting system by conventional chemotherapy, by the rescue of all the required physiological functions. Clinically, the rescued functions seemed to be responsible for the specific cell death in occult metastasis in the peripheral blood (prevent the recurrence and relapse of cancer), and for the recovery from anemia and hypoleukocytemia.

This could be typically observed in the results obtained in the patient No.12, by up-regulation of DEGs responsible for signal transfer in NF κ B and TGF- β signalling pathway²⁵, with further influences to related regulatory systems.

Immunological systems and pathways are multi-functional biosystems, and both deficiency as well as excessive functional enhancement cause various disorders and diseases. The results obtained in patient No.12 provided a hint for the major molecules and pathways. The continuous up-regulation of DEGs was observed which was responsible for signal transfer in NF κ B and TGF- β signalling pathway²⁵, whereas the other patients successfully recovered showed the down-regulation of these systems at a certain point of Huaier complementally therapy (**Fig. 3b**).

From the results obtained in the present study, we identified Huaier effects on the immunotolerance to the patients on the course of recovery from cancer, and that depended on the quantity and duration of administration, and also on the onset time of Huaier therapy. The sequential control of immunological reactions, both activation as well as modulation of excessive enhancement, were required for the recovery of cancer, by NF κ B and TGF- β signalling pathways.

In **extended Table 2** and **3**, we provided the copy number changes which might link to NF κ B by NF κ B subunits 1 and 2; molecules related to the production of iPS cells (KIT, Myc, OCT3/4, SOX2, LIN28A and NANOG). However, there observed no significant increase and/or decrease among patients.

Not only the genes correlating with signal transduction and carcinogenesis, many other genes of physiological importance should be also affected as a link to main pathways for maintenance of homeostasis. More importantly, almost all the transcriptional misregulation were rescued chiefly by the up-regulated transcription factors, with the down-regulation of N-CoR (**Fig. 3b**). Huaier effects on transcriptional control rescue has been reported before^{7,8}, and we frequently identified Hippo signaling pathway^{4,5}, Wnt signaling pathway rescue²² in the KEGG pathway²⁴ classification results in the present study.

The functional enrichment classified by KEGG pathway²⁴ specifically using the results of every TNM stages of colorectal cancer were shown in **Fig. 3c**. Blue box represents down-regulated genes and pathways, and red box indicates up-regulated ones. The panels were placed according to the stage of cancer progression, and the last panels showed the comparison of Huaier effects on benign adenoma vs. adenocarcinoma. The Patient No. 20 was a control without Huaier administration, with a latent progression of benign adenoma (dissected endoscopically one month after the final sampling). There were significantly more suppressive effects in the responsible oncogenes and tumour suppressors in benign adenoma cases than progressive adenocarcinoma cases. Details of relating signalling pathways were shown in **Fig. 3b**. The results obtained from the Patient No.20 indicated that, on the early process of cancer progression, the immunological pathways were down-regulated, whereas cell cycle signalling and oxidative stress accumulation were up-regulated.

Interestingly, the alterations in the oxidative stress reduction systems by Huaier were identical in different cancers, especially strong similarity found in hepatoma and meningioma (Patient No.8 and No.10 in **Fig. 3b**), which might suggest the required rescue system and/or functional damaging factors by stress accumulation were identical in those two organs, liver and central nervous system. The typical low responder to Huaier, the patient No.7, did not demonstrate any significant DEGs alterations in any pathways.

We analysed the relationship of those quantitative and qualitative DEG alterations to encoding transcription factors (TF). **Fig. 4a** showed transcription factor families and their quantitative changes classified by the DEGs which belonged to. Total 7,664 DEGs coding transcriptional factors were altered to rescue and/or modify transcriptional misregulation noted in **Fig. 3b**, within 30 days after Huaier administration. We have already indicated Huaier effects on the rescue of transcriptional control using *Drosophila* mutants⁵, and the present study provides the systematic and quantitative background. These dynamic changes in transcription factors might explain the enormous range of transcriptional control rescue by Huaier. As shown in **Table 4**, 66 kinds of transcription families were altered and modified by up/down regulation of encoding DEGs, maximum differences identified are 2,535 DEGs within 30 days, and 2,293 DEGS at 90 days after Huaier

administration. Again, we have to emphasise that these effects were observed with no side effects, toxicity, which resulted in the improvement of QOL of the patients.

The results of the changes of TF-DEG network were demonstrated in **Fig. 4b** as a linkage map of TF-cored transcripts, according to the time course of Huaier administration. The red and green dots represent the up-regulated and down-regulated DEGs, respectively. Purple ball represents each TF as a core. The greater the core size, the more DEGs regulated by the core transcription factor. The results indicated the extreme lineage of gene expression was evoked soon after the Huaier administration in cancer patients as shown in the former tables and figures, suggesting the genetic changes would be based on transcriptional control via small nuclear RNAs.

It is clearly demonstrated the extensive changes and rescues in transcriptional control induced by Huaier administration, with a variation of low-responder and high responders, with slow-responders and fast-responders. The recovery of cancer was easily identified to see the quantitative level of regulation in transcriptomes, with a confirmation of CT, MRI, and X-ray images of the patients (data not shown). However, there were many patients without significant gene expression changes. It is surprising to find no significant quantitative changes found specially in the patient with oesophageal cancer even with multiple metastasis (patient No.29).

In the present study, to investigate the exact information on transcriptional alterations as previously reported, the detailed simultaneously small non-coding RNAs (sncRNA) were sequenced using BGISEQ-500 technology^{30,31}. The numbers of detected small non-coding RNA for each sample were summarized in **Table 5**.

Before the data analysis, low-quality tags were removed from the samples (data filtering), and only used the samples of base quality with clean tags. In the end, length distribution analysis indicated that the length of small RNA is between 18 and 30 nucleotides. After filtering, clean tags were mapped to sRNA database such as miRBase³⁸, Rfam³⁹. To make unique small RNA mapped to only one annotation, we follow priority rule: miRNA>piRNA>Rfam>other sRNA. After sRNA annotation, those unknown tags will be used to predict novel sRNA based on their architectural features. Thus, novel miRNA, piRNA, and siRNA were predicted. **Table 6** showed the results of different kinds of sRNA in all the samples (the ratio of each components was shown in **extended Table 4**). The structure of miRNAs and the sequences of the novel small RNAs were deposited to the NCBI GEO (GSE157086), and shown and stored in *precursor.tar.gz*.

The small RNA expression level was calculated by using TPM⁴⁰ (Transcripts Per Kilobase Million). The analysed differentially expressed small RNAs (DESS) were designated to compare between samples. DESS in each pairwise were shown in **Fig. 5**. The changes in each patient by time course were demonstrated in **Fig. 6**.

In the patient No.5 (oesophageal squamous cell cancer with lung metastasis, we identified 20 up-regulation at 90 days after Huaier administration, and with one down-regulated element, total 21 novel elements were all down-regulated at 11 months after administration (**Figs. 6**). The slow onset of novel siRNA function was also noteworthy in this patient. Compared with the changes in transcriptomes (**Fig. 2**) and TF-DEG network (**Fig. 4b**), these siRNAs seemed to contribute to the drastic alterations of downregulation of the transcriptomes, followed by up-regulation of over 3,000 transcripts later. DEG changes were also associated with the drastic up-regulation of transcription factor families.

SiRNA has been reported to interfere with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation⁴¹. In addition, siRNAs typically work by cleaving the mRNA before translation, and have 100% complementarity, thus very tight target specificity⁴². These sequential genetic events found in patient No.5 in total would rescue the misregulation of translational and transcriptional systems *in vivo*, required for cancer recovery. In the other patients, no significant siRNA function was identified.

On the other hand, as shown in **Table 5**, average 1,003 miRNAs (ranging from 685 to 1,378) per sample were identified within 90 days after Huaier administration. DESs in each pair wise was shown in **Table 5**, **Figs. 5 and 6**, revealed several hundreds of miRNA alterations in each patient, and over a half of total identified miRNAs were differentially expressed. The comparison between **Figs. 2 and 6**, miRNA-mediating gene silencing effects were well demonstrated. For example, the significant changes in up- and down-regulated DEGs were in inverse proportion to those observed in quantitative DES changes, such as significant increase of up-regulated miRNAs in the patient No. 8 (**Fig. 6**, middle column) resulted in the significant decrease of both up- and down-regulated DEGs in **Fig. 2**. In contrast, the significant decrease of up-regulated DESs in **Fig. 6**, related to human papillomavirus infection suggested by **Fig. 3a**, drastically increased both up- and down-regulation of DEGs up to 3,330 and 3,505, respectively (12.2% of the total DEGs). The total numbers of DEG changes in the Patient No.8 became 85% of total transcripts (23,210/27,447).

In the patient 10, the increase of down-regulated miRNA was observed when massive increase of down-regulated DEGs (4,274; 15.6% of the total). In both patients No. 8 and 10, the opportunistic virus infection might influence the drastic decrease in number of miRNAs.

The present study also increased the information of the many novel miRNAs significantly contributed to cancer recovery, and further investigation of these molecules *in vitro* and *in vivo* are expected. MiRNAs have been reported to typically silence genes by repression of translation, and with broader specificity than siRNA, to functions in RNA silencing and post-transcriptional regulation of gene expression⁴²⁻⁴⁴.

In the present study, haemoglobin counts in TNM stage IV colorectal cancer improved from 6.0 to 12.0 g/dl after 4 months' treatment with 20g Huaier per day. MiRNAs also play crucial roles in the regulation of complex enzymatic cascades including the haemostatic blood coagulation system⁴⁵. Large scale studies of functional miRNA targeting have recently uncovered rationale therapeutic targets in the haemostatic system⁴⁶.

Piwi-interacting RNA (piRNA) is the largest class of small non-coding RNA molecules (containing about 24 nucleotides in the present study) expressed in animal cells⁴⁷. PiRNAs were reported distinct from microRNA (miRNA) in size (24 nucleotides as opposed to 21–22 nt), lack of sequence conservation, increased complexity. However, as shown in **Fig. 6**, the dynamic statistics of piRNA revealed similar movement as miRNAs, although the total numbers of the known and the novel piRNAs were much smaller compared with miRNAs. The present study, together with other small RNA information, shed lights into the function of piRNA function related to RNA-mediated adaptive immunity against transposon expansions and invasions, especially in the case with the opportunistic infection.

While many unusual expressions of long ncRNAs in disease states have been reported⁴⁸, it is still remained unknown how those ncRNAs function in causing diseases. Transcriptome analyses here identified numerous differentially expressed novel ncRNAs in various forms of cancer such as colorectal carcinoma and hepatocellular carcinoma. Many SNPs have been found to be associated with certain disease conditions, which function in association with targeting ncRNAs in the transcriptional regulation⁴⁸. All the novel small RNA (DSG) sequences identified were also deposited to the NCBI GEO (GSE157086).

In conclusion, the present study provided almost all the genomic and genetic events on cancer patients, especially identified from the recovered patients. The recovery from cancer was identical to the recovery of homeostasis, through the modulation and rescue of transcriptional dysregulation using the multi-elements involved in the series of each process in transcription and translation. SncRNAs together with dynamic RNA editing in SNP variation directly influenced these processes, which resulted in large-scale alterations in DEGs. Huaier effects could be quantitatively and widely observed in the biophysiological systems classified by Gene Ontology, the changes presented here in total were derived from the physiological capability and flexibility of each patient.

Thus, we have observed a striking molecular function as anti-cancer effects of Huaier. Those changes and modulations, in total, contributed every point required for the dynamic and systematic improvement of transcriptional control to recover biophysiological condition i.e., individual homeostasis.

Although there have been many reports regarding specific cancer cell death as anti-cancer effects of Huaier^{5, 12-23}, it should be emphasized the efficacy of Huaier on the significant potential for the recovery from opportunistic virus infections, and also for tissue reproduction to regenerate the damaged tissues and organs after the removal and/or cancer cells in the lesion. The elimination of occult metastasis (after surgical treatment) should be emphasized, too. These effects are spontaneous, and dose- and duration-dependent. It is the major reason why Huaier does not cause any side effects and/or toxicity.

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Authors' contributions T.T., M.T., designed the study from the clinical observation of the cancer patients with Huaier treatment (as a complementally therapy), and managed the sampling and clinical assessment of the patient volunteers, statistically analysed the data, and drafted the manuscript. F.T., H.L., C.X., managed total RNA and small nuclear RNA sequencing and conducted systematic analysis of the data. Z.L., D.W., contributed to the provision of Huaier granules and clinical evaluation of the data, especially focused on Immunological evaluation.

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Conflict of interest

The authors have no competing interest to declare.

Table 1. Clinical features of cancer patients and normal controls. The asterisks (*) of No.21 are the same as ones of No. 25. The double asterisks (**) of No. 5, 8 10 and 12 mean that the recovery from the opportunistic RNA virus infection could be clearly judged numerically by the methods hired in the present study.

Table 2. The numbers of RNA editing events identified in each sample. Left column summarizes the numbers by the classifications by SNP variation types, and right column for the classifications by splice event types.

Table 3. Quantitative analysis of Up- and Down-regulated differently expressed genes DEGs by time course of Huaier administration. The asterisks * indicate the time of opportunistic virus infection.

Table 4. Transcription Factor (TF) families classified by the DEGs which belonged to, and the numbers of DEGS up/down regulated by the time course of Huaier administration.

Table 5. The numbers of detected small non-coding RNA.

Table 6. The numbers of differentially expressed small RNAs (DSGs) in each patient by the time course of Huaier administration. a. numbers of differentially expressed siRNAs; b. the numbers of differentially expressed miRNAs; c. the numbers of differentially expressed piRNAs.

Extended Table 1. The comparison of the numbers of DEGs by the KEGG pathway classifications.

Extended Table 2. The comparison of NFκB subunits 1 (upper) and 2 (lower) copy numbers in all the samples.

Extended Table 3. The comparison of regulated DEGs (up- or down-) supposed to have intensive relationship to produce iPS cells (KIT, Myc, OCT3/4, SOX2, LIN28A and NANOG. a. comparison between before and 30 days after Huaier administration. b. comparison between before and 90 days after Huaier administration.

Extended Table 4. The resulting ratio (%) of analyzed different small non-coding RNA. To make every unique small RNA mapped to only one annotation, we follow the priority rule: miRNA>piRNA>snoRNA>Rfam>other small RNAs.

Figure Legends

Fig. 1. SNP variance after Huaier administration. **a.** classifications by SNP variation types; **b.** classifications by splice event types.

Fig. 2. Comparison of the numbers of differently expressed genes (DEGs) in each patient by time course of Huaier administration. The asterisks * indicate the time of opportunistic virus infection.

Fig. 3. Pathway classification and functional enrichment of DEGs. **a.** Pathway functional enrichment results for up/down regulation genes. X axis represents the terms of pathway. Y axis represents the number of up/down regulation genes. **b.** The detailed analysis of the Huaier effects on each cancer by KEGG biological pathways²⁵. The pathways responsible for cancer progression, stress reduction, and major immunological responses related to NF κ B and TGF β signalling pathway were specifically compared among patients with recovery and poor prognosis. **c.** Functional enrichment of the factors classified by KEGG pathways. The colour indicates the q-value (high: white, low: blue), the lower q-value indicates the more significant enrichment. Point size indicates DEG number (the bigger dots refer to larger amount). Rich Factor refers to the value of enrichment factor, which is the quotient of foreground value (the name of DEGs) and background value (total gene amount). The larger the value, the more significant enrichment.

Fig. 4. TF-DEG network in each patient by the time course of Huaier administration. **a.** TF families classified by the encoding DEGs, with each number of up/down regulation. **b.** TF-DEG predicted network. The red and green dots represent the up-regulated and down-regulated DEGs, respectively. Purple ball represents transcription factor, the greater the node the more DEGs the transcription factor regulate.

Fig. 5. Comparison of the numbers of DESs until 90 days of Huaier administration. Upper: Statistics of siRNA, middle: miRNA, lower: piRNA.

Fig. 6. Comparison of the numbers of DESs in each patient by time course of Huaier administration.

METHODS

Project Design, The present study initiated systematic understanding of the genomic and genetic requirement for the cancer recovery. Huaier compounds were provided by the manufacturer for this purpose with a strict control on transfer to Japan, good condition for maintenance, and provision to the patient volunteers.

The present study was strictly conducted according to the guidelines of the *Declaration of Helsinki* and the principles of good clinical practice. Written informed consent was obtained from the patients. This clinical research was applied according to the *Consolidated Standards of Clinical Research Trials* guidelines and was applied to the Japanese Medical Association on 9th February 2018, and approved on 5th March, 2018 (ID: JMA-IIA00335). The project has been strictly conducted with a monthly review by the ethics committee consisted by the experts on Medicine, Nursing, Laws, Pharmaceuticals, and Business Community (first committee held on 9th February, 2018).

We used Huaier compounds as complementary therapy, without any chemotherapy and radiotherapy which disrupt the molecular systems. Only surgical operation was performed when applicable. We thus planned and initiated an open-style, before-after controlled study, using peripheral blood as sampling materials to understand the almost all molecular events in each Huaier taking patient^{7,8}. The sampling materials were total blood, the same as reported previously^{7,8}, by the consideration of as follows; 1) easy to obtain from any candidate patients, 2) non- or less-hazardous procedure, and 3) applicable to any patients of any stages, even after the surgical operation. To compare with the other sampling, RNA extraction using nuclear cell components in peripheral blood rapidly reflects the biophysiological changes, and that more sensitively^{5,6} to monitor the course of any treatment than any other samples such as dissected organs.

Patient characteristics and sample collection. Basically, the volunteers were chosen on the demand of each individual, with a condition as written below;

- 1) diagnosed as cancer in the clinics,
- 2) exclusive for anti-cancer chemotherapy, radiotherapy, or any measures to influence genomic or genetic events in physiological condition for 90 days research period,
- 3) between ages of 35 to 85.

The normal healthy volunteers were chosen from the group of 1) without any diagnosed diseases, symptoms, and disorders, and 2) no drug administration, with an age distribution from ca. 35 to 85 years old.

The blood samples were obtained from the volunteer three times; 1: before, 2: 30 days after, and 3: 90 days after 20g per day Huaier administration^{14,23}. In case, more sampling was applied to some

patients for follow-up study in continuously Huaier taking patients. The samples (4ml blood) in PAXgene Blood RNA Tube (QIAGEN, Valencia, CA) was handled and kept at -80 °C until shipping to BGI Shenzhen by dry-ice cargo. The RNA extraction, quality check for sequencing were performed in BGI Shenzhen according to the BGI protocols.

For details regarding to the following procedures, the information is provided by website ; <http://www.bgitechsolutions.com/>.

RNA extraction & miRNA library construction. Total RNA was extracted in BGI Shenzhen from transferred total blood samples using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manual instructions. The mix was centrifuge at 12000×g for 5min at 4°C. The supernatant was transferred to a new 2.0ml tube which was added 0.3ml of Chloroform/isoamyl alcohol (24:1) per 1.5ml of Trizol reagent. After the mix was centrifuged at 12,000×g for 10min at 4°C, the aqueous phase was transferred to a new 1.5mL tube which was add equal volume of supernatant of isopropyl alcohol. The mix was centrifuged at 12,000×g for 20min at 4°C and then removed the supernatant. After washed with 1ml 75% ethanol, the RNA pellet was air-dried in the biosafety cabinet and then dissolved by add 25µL~100µL of DEPC-treated water. Subsequently, total RNA was qualified and quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA).

mRNA Library Construction. Oligo(dT)-attached magnetic beads were used to purified mRNA. Purified mRNA was fragmented into small pieces with fragment buffer at appropriate temperature. Then First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis. afterwards, A-Tailing Mix and RNA Index Adapters were added by incubating to end repair. The cDNA fragments obtained from previous step were amplified by PCR, and products were purified by Ampure XP Beads, then dissolved in EB solution. The product was validated on the Agilent Technologies 2100 bioanalyzer for quality control. The double stranded PCR products from previous step were heated denatured and circularized by the splint oligo sequence to get the final library. The single strand circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 to make DNA nanoball (DNB) which had more than 300 copies of one molecular, DNBs were loaded into the patterned nanoarray and single end 50 bases reads were generated on BGISEQ500 platform (BGI-Shenzhen, China).

Library was prepared with 1 µg total RNA for each sample. Total RNA was purified by electrophoretic separation on a 15% urea denaturing polyacrylamide gel electrophoresis (PAGE) gel and small RNA regions corresponding to the 18–30 nt bands in the marker lane (14-30 ssRNA Ladder Marker, TAKARA, Japan) were excised and recovered. Then the 18–30 nt small RNAs were ligated to a 5'-adaptor and a 3'-adaptor. The adapter-ligated small RNAs were subsequently transcribed into cDNA by SuperScript II Reverse Transcriptase (Invitrogen, USA) and then several rounds of PCR

amplification with PCR Primer Cocktail and PCR Mix were performed to enrich the cDNA fragments. The PCR products were selected by agarose gel electrophoresis with target fragments 100~120 bp, and then purified by QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The library was quality and quantitated in two methods: check the distribution of the fragments size using the Agilent 2100 bioanalyzer, and quantify the library using real-time quantitative PCR (QPCR) (TaqMan Probe). The final ligation PCR products were sequenced using the BGISEQ-500 platform (BGI-Shenzhen, China).

Transcriptome Resequencing. We first removed the reads mapped to rRNAs and get raw data, then we filtered the low quality reads (More than 20% of the bases qualities are lower than 10), reads with adaptors and reads with unknown bases (N bases more than 5%) to get the clean reads. Then we map those clean reads onto reference genome(hg38_UCSC_20180118), followed with novel gene prediction, SNP & INDEL calling and gene splicing detection. Finally, we identify DEGs (differentially expressed genes) between samples and do clustering analysis and functional annotations.

In the transcriptome analysis, all samples had library construction and sequenced on the MGISEQ-2000 and BGISEQ-500 platform. Paired-end reads were aligned to UCSC hg38 using Hisat2 and Bowtie2. We calculated the expression of genes by RSEM, call DEGs by PossionDis method, predicted novel transcripts by StringTie and call SNP by GATK .

Small RNA analysis. In the smallRNA analysis, all samples had library construction and sequenced on the BGISEQ-500 platform. Singled-end reads were aligned to UCSC hg38 MirBase22 and Rfam by Bowtie2. We calculated the expression of miRNAs by in-house program, predicted miRNA by mirdeep2, and predicted target genes by miRanda and TargetScan. The following processes were applied to the subsequent data analysis. a. Eliminate the low-quality reads, small tags, adaptors and other contaminants to get clean reads. b. Summarize the length distribution of the clean tags, common and specific sequences between samples. c. Annotate the clean tags into different categories. d. Predict the novel miRNA by exploring the characteristic hairpin structure of miRNA precursor. e. Function annotation of known miRNAs, including Gene Ontology and Pathway.

The detailed protocols were provided and demonstrated at BGI website:

<http://www.bgitechsolutions.com/>). The obtained novel genes and small nuclear non-coding RNA obtained in the present study have been deposited to The NCBI GEO (GSE157086).

References

1. Zauber A. G. *et al.* Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. *N. Engl. J. Med.* **366**, 687-696 (2012).
2. Okada, K., Sadahiro, S., Ogimi, T. *et al.* Tattooing improves the detection of small lymph node and increases the number of retrieved lymph nodes in patients with rectal cancer who receive preoperative chemoradiotherapy: A randomized controlled clinical trial. *Am. J. Surgery*, **215**, 563-569 (2017).
3. Peng, Z. *et al.* Comprehensive analysis of RNA-Seq data reveals extensive RNA editing in a human transcriptome. *Nat. Biotechnol.* **30**, 253–260 (2012).
4. Mo, J. S., Park, J. W. & Guan, K. L. The Hippo signaling pathway in stem cell biology and cancer. *EMBO Rep.* **25**, 642-656 (2014).
5. Tanaka, T. *et al.* Huaier Regulates Cell Fate by the Rescue of Disrupted Transcription Control in the Hippo Signaling Pathway. *Arch. Clin. Biomed. Res.* **1**, 179–199 (2017).
6. Tanaka, M. *et al.* Rapid and quantitative detection of a mammalian septin Bradeion as a practical diagnostic method of colorectal and urologic cancers. *Med. Sci. Monit.* **9**, 61-68 (2003).
7. Jiang, Y. *et al.* Proteomics identifies new therapeutic targets of early-stage hepatocellular carcinoma. *Nature*, **567**, 257–261 (2019).
8. Song, Y. *et al.* Identification of genomic alterations in oesophageal squamous cell cancer. *Nature* **509**, 91–95 (2014).
9. Chongtham, A. & Agrawal, N. Crucumin modulates cell death and its protective in Huntington's disease model. *Sci. Rep.* **6**, 18736-18745 (2016).
10. Da Rocha, A. B., Lopes, R. M. & Schwartzmann, G. Natural products in anticancer therapy. *Curr. Opin. Pharmacol.* **1**, 364-369 (2001).
11. Li L, Ye S, W. Y. Progress on experimental research and clinical application of *Trametes robiniophila*. *Bull. Chin. Cancer* **16**, 110–113 (2007).
12. Huo, Q. & Yang, Q. Role of Huaier Extract as a Promising Anticancer Drug. *Adapt. Med.* **4**, 1–6 (2012).
13. Sun, Y. *et al.* A polysaccharide from the fungi of Huaier exhibits anti-tumor potential and immunomodulatory effects. *Carbohydr. Polym.* **92**, 577-582 (2013).
14. Wang, X. *et al.* Chinese medicines for prevention and treatment of human hepatocellular carcinoma: current progress on pharmacological actions and mechanisms. *J. Integr. Med.* **13**, 142–164 (2015).
15. Wang, X. *et al.* Huaier aqueous extract inhibits stem-like characteristics of MCF7 breast cancer cells via inactivation of hedgehog pathway. *Tumor Biol.* **35**, 10805-10813 (2014).

16. Li, C. *et al.* A Huaier polysaccharide inhibits hepatocellular carcinoma growth and metastasis. *Tumour Biol: J. Int. Soc. Oncodevelopmental. Biol. Med.* **36**, 1739–1745 (2015).
17. Zheng, J. *et al.* Huaier polysaccharides suppresses hepatocarcinoma MHCC97-H cell metastasis via inactivation of EMT and AEG-1 pathway. *Int. J. Biol. Macromol.* **64**, 106–110 (2014).
18. Chen, Q. *et al.* Effect of Huaier granule on recurrence after curative resection of HCC: A multicentre, randomised clinical trial. *Gut* **67**, 2006–2016 (2018).
19. Wu, T. *et al.* Huaier suppresses proliferation and induces apoptosis in human pulmonary cancer cells via upregulation of miR-26b-5p. *FEBS Lett.* **588**, 2107–2114 (2014).
20. Yan, X. *et al.* (2013) Huaier aqueous extract inhibits ovarian cancer cell motility via the AKT/GSK3 β /beta-catenin pathway. *Plos One* **8**, e63731.
21. Zhang, T. *et al.* Huaier aqueous extract inhibits colorectal cancer stem cell growth partially via downregulation of the Wnt/ β -catenin pathway. *Oncol Lett.* **5**, 1171–1176 (2013).
22. Cui, Y., Meng, H., Liu, W., Wang, H., & Liu, Q. Huaier aqueous extract induces apoptosis of human fibrosarcoma HT1080 cells through the mitochondrial pathway. *Oncol Lett.* **9**, 1590–1596 (2015).
23. Song, X., Li, Y., Zhang, H. & Yang, Q. The anticancer effect of Huaier (Review). *Oncol. Rep.* **34**, 12–21 (2015).
24. Kanehisa, M. *et al.* KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* **36**, 480–484 (2008).
25. Ramsköld, D. *et al.* Full-Length mRNA-Seq from single cell levels of RNA and individual circulating tumor cells. *Nat Biotechnol* **30**, 777–782 (2013).
26. Cock, P. J. A., Fields, C. J., Goto, N., Heuer, M. L. & Rice, P. M. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. *Nucleic Acids Res.* **38**, 1767–1771 (2009).
27. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements Daehwan HHS Public Access. *Nat. Methods* **12**, 357–360 (2015).
28. Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for Transcriptomics. **10**, 57–63 (2010).
29. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* **5**, 621–628 (2008).
30. Robinson, J. T. *et al.* Integrative Genome Viewer. *Nat. Biotechnol.* **29**, 24–6 (2011).
31. Pertea, M. *et al.* StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* **33**, 290–295 (2015).

32. Trapnell, C. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578 (2013).
33. Kong, L. *et al.* CPC: Assess the protein-coding potential of transcripts using sequence features and support vector machine. *Nucleic Acids Res.* **35**, 345–349 (2007).
34. DePristo, M. *et al.* A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet.* **43**, 491–498 (2011).
35. Shen, S. *et al.* rMATS: Robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E5593–E5601 (2014).
36. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **10**, (2009).
37. Li, B. & Dewey, C. N. RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, (2011).
38. Griffiths-Jones, S. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* **34**, D140–D144 (2006).
39. Nawrocki, E. P. *et al.* Rfam 12.0: Updates to the RNA families database. *Nucleic Acids Res.* **43**, D130–D137 (2015).
40. Li, B., Ruotti, V., Stewart, R. M., Thomson, J. A. & Dewey, C. N. RNA-Seq gene expression estimation with read mapping uncertainty. *Bioinformatics* **26**, 493–500 (2009).
41. Laganà, A. *et al.* Computational design of artificial RNA molecules for gene regulation. *Methods Mol. Biol.* **1269**, 393–412 (2015).
42. Mack, G. S. MicroRNA gets down to business. *Nat. Biotechnol.* **25**, 631–638 (2007).
43. Fabian, M. R., Sonenberg, N. & Filipowicz, W. Regulation of mRNA Translation and Stability by microRNAs. *Annu. Rev. Biochem.* **79**, 351–379 (2010).
44. Bartel, D. P. MicroRNAs: Target Recognition and Regulatory Functions. *Cell* **136**, 215–233 (2009).
45. Teruel-Montoya, R., Rosendaal, F. R. & Martínez, C. MicroRNAs in hemostasis. *J. Thrombosis and Haemostasis*, **13**: 170–181 (2015).
46. Nourse, J., Braun, J., Lackner, K., Hüttelmaier, S. & Danckwardt, S. Large-scale identification of functional microRNA targeting reveals cooperative regulation of the hemostatic system. *J. Thrombosis and Haemostasis*, **16**, 2233–2245 (2018).
47. Seto, A. G., Kingston, R. E., & Lau, N. C. The Coming of Age for Piwi Proteins. *Mol. Cell*, **26**, 603–609 (2007).
48. Ma, L. *et al.* Lncbook: A curated knowledgebase of human long non-coding rnas. *Nucleic Acids Res.* **47**, D128–D134 (2019).

Table 1

Patient			diagnosis	stage	metastasis	Sampling time after Huaier administration										complication	additional treatment	prognosis
No.	age	gender				before	30 days	90days	6 months	9 months	11 months	12 months	14 months	18 months				
1	70	M	lung cancer (small cell carcinoma)	IV	lymph nodes, liver	○	○	○							pleural effusion, anemia	chemotherapy for 1 year	deceased (144days)	
2	67	F	pancreas cancer	IV	liver, lumbar vertebrae	○	-	-							not specified	chemotherapy for 4 months + radiotherapy	deceased (35 days)	
3	70	M	pancreas cancer	IV	peritoneal metastasis	○	○	○							lung, severe anemia	chemotherapy for 4 months + radiotherapy	deceased (120days)	
4	67	M	cholangiocarcinoma	IIb	liver	○	○	○							jaundice	traditional Chinese medicine complex	deceased (10 months)	
5	72	M	oesophageal squamous cell cancer	IIb	lung	○	○	○*			○		○	○	not specified	chemotherapy for 4 months + radiotherapy	remission	
6	68	M	stomach cancer (signet ring cell ca.)	IIa	not detected	○	-	-							not specified	Vitamin C injection	deceased (35 days)	
7	56	M	colorectal cancer	IV	lymph nodes, lung	○	○	-							diabetes mellitus(type II)	chemotherapy for 1 year	deceased (140days)	
8	78	M	hepatoma	I	pancreas(suspected)	○	○	○	○	○*				○	hepatitis C (Huminant)	Interleukin alfa + liver treatment	remission	
9	67	M	oesophageal squamous cell cancer	I	hypopharynx	○	○	○							hyperlipidemia	radiotherapy for 35 days	remission	
10	39	F	meningioma (benign)	-	-	○	○	○		○				○*	○	not specified	surgical dissection at 6 weeks after Huaier tr.	complete recovery
11	76	M	oesophageal adenocarcinoma	-	-	○	○	○								adenoma in stomach	surgical dissection before Huaier tr.	complete recovery
12	71	F	lung cancer (adenocarcinoma)	IV	brain, vertebrae	○	○	○*								anemia, pleural effusion	surgical dissection + chemotherapy	deceased (150days)
13	61	M	colorectal adenoma (benign)	-	-	○	○	○								not specified	endoscopic polyp dissection after Huaier tr.	complete recovery
14	61	F	colorectal adenoma (benign)	-	-	○	○	○								hepatoma (suspected)	endoscopic polyp dissection after Huaier tr.	complete recovery
15	73	M	prostate cancer (in situ (biopsy)	0	-	○	○	○								liver dysfunction	None	complete recovery
16	60	M	prostate cancer (in situ (biopsy)	0	-	○	○	○								liver dysfunction	None	complete recovery
17	62	M	stomach cancer (adenocarcinoma)	IV	lung, liver	○	○	○								anemia	chemotherapy followed	deceased (150 days)
18	63	M	lung cancer suspected by CT	0	-	○	○	○								prostate hyperplasia	None	complete recovery
19	70	F	colorectal cancer	IIb	lymph nodes	○	○	○								not specified	endoscopic dissection at 2 weeks after Huaier tr.	remission
20	70	F	colorectal adenoma (benign)	-	-	○	○	○								hypertension	Huaier-free control, endoscopic dissection	complete recovery
21	66	M	healthy control	-	-	○	○	○				○*	○*	○*		not specified	None	healthy
22	73	F	breast cancer	IV	lymph nodes, lung, liver	○	○	○								anemia, anorexia	None	deceased (93 days)
23	61	F	breast cancer	IV	lymph nodes, lung, liver	○	-	-								anemia, anorexia	surgical dissection planned	deceased by accident
24	72	M	cholangiocarcinoma	IV	lung, liver, prostate	○	○	-								anemia, anorexia	chemotherapy for 4 months + radiotherapy	deceased (58 days)
25	66	M	healthy control	-	-	○*	○*	○*								not specified	None	healthy
26	73	M	healthy control	-	-	○	○	○*								prostate hyperplasia	None	healthy
27	61	M	prostate cancer	I	-	○	○	○								not specified	surgical dissection before Huaier tr.	remission
28	64	F	oesophageal squamous cell cancer	II	lymph nodes	○	○	○*								multi-cancer suspected	endoscopic dissection before Huaier tr.	remission
29	83	F	oesophageal squamous cell cancer	IV	lung	○	○	○*								dysphagia	endoscopic dissection before Huaier tr.	remission
30	72	M	pancreas cancer	IV	lung, liver, vertebrae	○	○	○*								anemia, anorexia, ascites	chemotherapy for 4 months	deceased (60 days)
31	72	M	pancreas cancer	IV	lung, liver, vertebrae	○	○	○*								anemia, anorexia, ascites	chemotherapy for 6 months+ immunotherapy	deceased (120 days)

Table 2

Sample	Skipped exons	Mutually exclusive exons	Alternative 5' splice sites	Alternative 3' splice sites	Retained introns
KS1_1	12,633	1,975	2,820	3,453	4,317
KS1_2	12,935	2,023	2,865	3,497	4,346
KS1_3	13,415	2,062	2,777	3,615	4,448
KS2_1	10,520	1,700	2,211	2,989	3,973
KS3_1	13,561	2,160	2,881	3,498	4,343
KS3_2	12,212	1,868	2,559	3,341	4,287
KS3_3	12,927	2,103	2,608	3,437	4,338
KS4_1	15,222	2,366	2,957	3,801	4,603
KS4_2	15,323	2,432	2,887	3,766	4,485
KS4_3	17,577	2,887	3,154	3,959	4,667
KS5_1	12,774	2,081	2,787	3,777	4,873
KS5_2	13,852	2,352	2,914	3,902	4,968
KS5_3	18,120	3,014	3,589	4,624	5,451
KS5_4	16,227	2,883	3,326	3,896	4,788
KS5_5	10,747	1,702	2,391	3,195	4,434
KS5_6	11,740	1,851	2,522	3,421	4,564
KS6_1	14,622	2,316	2,851	3,732	4,543
KS6_2	12,488	1,936	2,546	3,465	4,290
KS7_1	10,993	1,857	2,211	2,991	3,921
KS7_2	11,537	1,947	2,349	3,083	4,071
KS8_1	16,009	2,647	3,358	4,342	5,300
KS8_2	18,702	3,088	3,704	4,629	5,460
KS8_3	15,285	2,495	3,297	4,192	5,297
KS8_4	17,751	2,900	3,573	4,673	5,495
KS8_5	17,177	2,986	3,406	4,098	4,930
KS8_6	14,582	2,240	2,957	3,888	4,887
KS9_1	14,382	2,194	2,762	3,674	4,514
KS9_2	14,635	2,273	2,835	3,686	4,562
KS9_3	12,535	1,985	2,625	3,393	4,444
KS10_1	15,393	2,510	3,235	4,241	5,272
KS10_2	16,385	2,689	3,370	4,405	5,366
KS10_3	11,826	1,964	2,643	3,435	4,752
KS10_4	18,428	3,258	3,567	4,255	4,968
KS10_5	12,485	1,949	2,899	3,588	4,715
KS10_6	12,917	1,958	2,541	3,527	4,624
KS11_1	14,220	2,245	2,865	3,701	4,546
KS11_2	16,731	2,639	3,048	4,022	4,742
KS11_3	11,624	1,791	2,475	3,234	4,254
KS12_1	10,686	1,720	2,336	3,091	4,196
KS12_2	12,663	1,922	2,623	3,395	4,402
KS12_3	19,240	3,092	3,545	4,519	5,309
KS13_1	11,896	1,904	2,513	3,249	4,296
KS13_2	11,674	1,847	2,527	3,252	4,321
KS13_3	18,317	3,111	3,476	4,417	5,254
KS14_1	12,044	1,900	2,541	3,321	4,324
KS14_2	11,072	1,774	2,394	3,157	4,196
KS14_3	17,198	2,760	3,342	4,237	5,119
KS15_1	12,475	1,901	2,634	3,469	4,413
KS15_2	18,053	2,917	3,407	4,358	5,238
KS15_3	17,762	2,992	3,385	4,357	5,231
KS16_1	13,103	2,142	2,722	3,476	4,431
KS16_2	17,066	2,797	3,306	4,284	5,231
KS16_3	16,040	2,636	3,202	4,117	5,105
KS17_1	14,215	2,237	2,800	3,663	4,497
KS17_2	17,502	2,844	3,338	4,279	5,176
KS17_3	17,730	2,901	3,443	4,329	5,199
KS18_1	13,401	2,095	2,703	3,525	4,470
KS18_2	17,753	2,945	3,363	4,255	5,232
KS18_3	16,334	2,606	3,210	4,158	5,122
KS19_1	12,627	1,976	2,889	3,413	4,400
KS19_2	18,471	3,063	3,510	4,421	5,256
KS19_3	17,534	2,852	3,377	4,342	5,264
KS20_1	17,427	2,820	3,449	4,415	5,633
KS20_2	18,082	2,934	3,577	4,584	5,734
KS20_3	17,442	2,990	3,451	4,065	5,177
KS21_1	18,735	3,119	3,891	4,699	5,835
KS21_2	18,089	2,955	3,606	4,544	5,758
KS21_3	16,511	2,908	3,342	4,095	4,956
KS22_1	16,179	2,815	3,232	4,254	5,433
KS22_2	16,133	2,907	3,192	3,834	4,977
KS22_3	13,438	2,395	2,726	3,377	4,570
KS24_1	15,205	2,711	3,048	3,736	4,718
KS24_2	12,298	2,055	2,491	3,457	4,549
KS25_1	15,246	2,425	3,037	4,052	5,116
KS25_2	13,651	2,163	2,824	3,705	4,745
KS25_3	9,768	1,521	2,182	2,984	4,174
KS26_1	10,311	1,638	2,339	3,124	4,320
KS26_2	12,071	1,968	2,450	3,284	4,388
KS26_3	11,753	1,805	2,497	3,418	4,503
KS27_1	12,121	1,908	2,555	3,453	4,563
KS27_2	9,849	1,601	2,133	2,936	4,094
KS27_3	11,910	1,931	2,538	3,362	4,520
KS28_1	13,399	2,083	2,767	3,688	4,825
KS28_2	11,134	1,741	2,481	3,366	4,560
KS28_3	12,818	1,902	2,710	3,546	4,695
KS29_1	9,946	1,516	2,200	2,973	4,200
KS29_2	10,666	1,667	2,333	3,106	4,334
KS29_3	12,686	1,944	2,674	3,524	4,654
KS30_1	10,639	1,637	2,309	3,115	4,302
KS30_2	8,795	1,351	1,997	2,803	4,030
KS31_1	7,657	1,229	1,655	2,341	3,412
KS31_2	14,796	2,436	2,931	3,795	4,847
Total	1,303,783	211,336	264,368	343,124	435,142
Average	14,172	2,297	2,874	3,730	4,730
Max	19,240	3,258	3,704	4,699	5,835
Min	7,657	1,229	1,655	2,341	3,412

Sample	A-G	C-T	Transition	A-C	A-T	C-G	G-T	Transversion	Total
KS1_1	27,269	27,034	54,303	4,598	3,092	6,200	4,659	18,549	72,852
KS1_2	29,664	29,625	59,289	5,093	3,570	6,664	5,155	20,462	79,771
KS1_3	30,485	30,057	60,542	5,162	3,462	6,895	5,199	20,758	81,300
KS2_1	28,184	27,953	56,137	4,881	3,388	6,113	4,761	19,143	75,280
KS3_1	28,505	28,393	56,898	4,947	3,423	6,688	4,938	19,996	76,894
KS3_2	27,712	27,633	55,345	4,719	3,141	6,344	4,760	18,964	74,309
KS3_3	28,275	28,022	56,297	4,765	3,161	6,506	4,849	19,281	75,578
KS4_1	36,375	36,491	72,866	6,186	4,226	8,137	6,173	24,722	97,588
KS4_2	35,590	35,178	70,768	6,130	4,301	7,948	6,182	24,561	95,329
KS4_3	36,892	36,941	73,833	6,369	4,596	8,350	6,424	25,739	99,572
KS5_1	25,825	25,597	51,422	4,389	3,122	5,997	4,515	18,023	69,445
KS5_2	27,696	27,980	55,676	4,795	3,397	6,351	4,961	19,504	75,180
KS5_3	39,988	39,975	79,963	6,905	5,193	9,208	6,998	28,304	108,267
KS5_4	35,477	35,332	70,809	5,968	4,635	7,651	6,196	24,450	95,259
KS5_5	22,091	21,880	43,971	4,399	3,151	5,381	4,234	17,165	61,136
KS5_6	22,635	22,337	44,972	4,338	3,272	5,377	4,184	17,171	62,143
KS6_1	34,169	33,696	67,865	5,860	3,996	7,595	5,713	23,164	91,029
KS6_2	28,918	28,489	57,407	4,951	3,397	6,377	4,905	19,630	77,037
KS7_1	27,082	26,902	53,984	4,486	3,152	5,843	4,605	18,086	72,070
KS7_2	28,315	28,919	57,234	4,719	3,330	6,268	4,762	19,079	76,313
KS8_1	33,089	32,820	65,909	5,539	3,819	7,516	5,573	22,447	88,356
KS8_2	46,621	45,998	92,619	7,997	6,005	9,990	7,954	31,946	124,565
KS8_3	39,089	38,915	78,004	7,244	5,460	8,994	7,381	29,079	107,083
KS8_4	48,001	48,049	96,050	7,468	5,514	10,046	7,721	30,749	126,799
KS8_5	38,094	37,786	75,880	6,508	5,044	7,995	6,497	26,044	101,924
KS8_6	32,377	31,778	64,155	6,135	4,689	7,556	5,890	24,270	88,425
KS9_1	34,213	34,018	68,231	5,876	4,125	7,724	5,705	23,430	91,661
KS9_2	33,480	33,400	66,880	5,640	4,048	7,445	5,525	22,658	89,538
KS9_3	30,875	30,871	61,746	5,649	3,874	7,381	5,700	22,604	84,350
KS10_1	35,699	35,402	71,101	5,956	4,138	7,819	5,866	23,779	94,880
KS10_2	38,584	38,318	76,902	6,626	4,944	8,555	6,480	26,715	103,617
KS10_3	27,475	27,433	54,908	5,135	3,927	6,495	5,435	21,272	76,180
KS10_4	44,956	45,029	89,985	7,499	5,637	9,296	7,565	30,197	120,182
KS10_5	27,704	27,533	55,237	5,166	3,774	6,973	4,970	20,583	75,820
KS10_6	26,403	26,226	52,629	5,010	3,818	6,341	4,873	20,042	72,671
KS11_1	35,106	35,126	70,232	5,795	3,987	7,748	5,798	23,328	93,560
KS11_2	39,922	39,628	79,550	6,836	4,974	8,868	6,580	27,258	106,806
KS11_3	35,839	35,902	71,741	7,779	6,345	8,429	7,819	30,372	102,113
KS12_1	27,510	27,409	54,919	5,294	3,725	6,689	5,345	21,053	75,972
KS12_2	35,134	35,049	70,183	6,964	5,014	8,311	6,789	27,078	97,261
KS12_3	55,119	55,174	110,293	8,861	6,305	11,648	8,995	35,809	146,102
KS13_1	29,132	29,286	58,418	5,758	4,048	7,057	5,783	22,646	81,064
KS13_2	27,996	27,911	55,907	5,470	3,792	6,709	5,556	21,527	77,434
KS13_3	43,614	43,369	86,983	6,905	4,761	9,382	7,033	28,081	115,064
KS14_1	33,015	32,764	65,779	6,542	4,787	7,510	6,519	25,358	91,137
KS14_2	28,324	27,978	56,302	5,352	3,848	6,611	5,332	21,143	77,445
KS14_3	51,175	51,497	102,672	8,268	5,811	10,522	8,239	32,840	135,512
KS15_1	35,232	35,381	70,613	6,959	5,347	7,974	6,805	27,085	97,698
KS15_2	44,555	44,772	89,327	7,142	5,156	9,486	7,152	28,936	118,263
KS15_3	47,501	47,843	95,344	7,479	5,545	9,955	7,652	30,631	125,975
KS16_1	32,430	32,311	64,741	5,734	4,308	7,107	5,791	22,940	87,681
KS16_2	47,450	47,705	95,155	7,665	5,529	9,850	7,587	30,631	125,786
KS16_3	40,282	40,706	80,988	6,480	4,619	8,441	6,515	26,055	107,043
KS17_1	36,985	37,179	74,164	6,534	4,642	8,299	6,686	26,161	100,325
KS17_2	40,056	39,674	79,730	6,445	4,622	8,664	6,456	26,217	105,947

Table 3

Huaier administratio n	30 days		90 days		6 months		9 months		11 months		1 year		14 months		1.5 year		Total	
Patient No.	up	down	up	down	up	down	up	down	up	down	up	down	up	down	up	down	Up	down
1	954	434	58	63													1,012	497
2	deseased after 1st sampling																	
3	303	723	528	1,134													831	1,857
4	512	113	319	259													831	372
5	67	28	1,750	105					2,351	3,351			3,468*	2,847	147	560	7,783	6,891
6	35	121															35	121
7	67	36															67	36
8	2,877	554	358	791	720	850	3,330*	3,505					3,533	2,891			10,818	8,591
9	47	225	1,005	471													1,052	696
10	1,799	812	810	4,247			5,671*	2,477					3,540	3,439	215	200	12,035	11,175
11	1,523	48	169	857													1,692	905
12	549	125	1,826*	353													2,375	478
13	36	53	1,119	505													1,862	558
14	66	94	1,685	1,178													1,751	1,272
15	1,455	1,141	60	47													1,515	1,188
16	1,166	736	35	186													1,201	922
17	313	239	140	442													453	681
18	664	498	62	183													726	681
19	1,580	823	26	186													1,606	1,009
20	21	30	3,746	3,103													3,767	3,133
21	204	82	1,424	2,746					3,094*	2,037	68	71	97	196			4,887	5,132
22	830	1,557	89	78													919	1,635
24	2,615	3,031															2,615	3,031
25	68	71	97	196													165	267
26	534	2,430	2,235	460													2,769	2,890
27	147	281	215	56													2,769	2,890
28	39	46	103	73													2,769	2,890
29	224	330	319	120													2,769	2,890
30	157	1,548															2,769	2,890
31	1,251	351															1,251	351
Average																	2,589	2,273
Max																	12,035	11,175
Mini																	35	36

Table 4

TF name	30 days	90 days
AF-4	56	52
AP-2	3	2
ARID	240	220
bHLH	150	165
C/EBP	10	9
CBF		3
CG-1	20	22
COE	7	4
CP2	12	9
CSD	11	9
CSL	1	0
CTF/NFI	12	10
CUT	86	98
DM	1	3
E2F	34	25
ESR-like	33	23
ETS	61	43
Fork	138	118
GCM	1	3
GCNF-like	4	2
GTF2I	19	22
HMG	272	271
HMG/HMGY	100	79
Homeobox	730	665
HPD	3	3
HSF	23	24
HTH	4	5
IRF	21	18
MBD	131	117
MH1	16	20
MYB	184	175
NCU-G1	2	2
NDT80/PhoG	4	5
NF-YA	1	2
NF-YB	9	5
NF-YC	14	13
NGFIB-like	14	7
Nrf1	1	3
Others	1,128	1,037
P53	7	11
PAX	24	9
PC4	11	6
Pou	51	34
RFX	22	15
RHD	146	105
Runt	9	9
RXR-like	13	9
SAND	32	21
SRF	18	16
STAT	24	15
T-box	20	17
TEA	3	2
TF_bZIP	158	116
TF_Otx	12	6
THAP	39	22
THR-like	37	35
TSC22	13	10
Tub	17	16
ZBTB	342	333
zf-BED	16	14
zf-C2H2	2,535	2,293
zf-C2HC	415	451
zf-GATA	101	96
zf-LITAF-like	4	4
zf-MIZ	26	26
zf-NF-X1	13	4
Total	7,664	6,988
Max	2,535	2,293
Min	1	0

Table 5

Sample name	Known miRNA count	Novel miRNA count	Known piRNA count	Novel piRNA count	Known siRNA count	Novel siRNA count
KS1_1	1,001	317	48	213	0	2
KS1_2	1,044	251	60	167	0	1
KS1_3	985	262	69	170	0	1
KS2_1	941	161	74	99	0	0
KS3_1	923	190	68	88	0	1
KS3_2	903	182	69	97	0	0
KS3_3	978	260	86	148	0	2
KS4_1	993	214	87	144	0	1
KS4_2	941	180	88	112	0	1
KS4_3	1,005	129	70	108	0	0
KS5_1	946	317	61	194	0	0
KS5_2	919	255	69	162	0	1
KS5_3	962	191	86	125	0	19
KS5_4	1,000	108	118	108	0	0
KS5_5	708	26	35	24	0	0
KS5_6	906	64	72	71	0	0
KS6_1	989	260	67	174	0	2
KS6_2	996	289	63	168	0	1
KS7_1	983	201	63	125	0	0
KS7_2	970	259	59	124	0	0
KS8_1	956	238	69	148	0	1
KS8_2	946	218	73	141	0	0
KS8_3	729	15	73	21	0	0
KS8_4	1,018	205	81	168	0	1
KS8_5	914	124	78	106	0	0
KS8_6	671	14	43	14	0	0
KS9_1	905	273	50	189	0	0
KS9_2	986	240	74	145	0	0
KS9_3	721	30	64	35	0	1
KS10_1	995	281	57	159	0	0
KS10_2	980	207	73	124	0	0
KS10_3	769	21	67	27	0	0
KS10_4	891	112	68	58	0	1
KS10_5	848	51	30	45	0	0
KS10_6	784	41	31	39	0	0
KS11_1	1,054	324	60	230	0	2
KS11_2	970	206	92	129	0	0
KS11_3	818	39	82	55	0	1
KS12_1	741	14	67	25	0	1
KS12_2	782	16	78	35	0	1
KS12_3	943	181	81	123	0	1
KS13_1	908	34	76	46	0	3
KS13_2	773	40	66	57	0	0
KS13_3	982	153	91	138	0	0
KS14_1	728	16	45	17	0	0
KS14_2	809	51	63	50	0	0
KS14_3	967	151	120	135	0	0
KS15_1	781	19	71	27	0	1
KS15_2	972	127	86	99	0	0
KS15_3	935	127	72	102	0	1
KS16_1	829	62	62	51	0	0
KS16_2	942	173	73	116	0	0
KS16_3	969	186	73	168	0	1
KS17_1	981	244	62	138	0	0
KS17_2	1,015	167	81	131	0	1
KS17_3	956	110	88	83	0	0
KS18_1	684	24	47	22	0	0
KS18_2	920	106	75	115	0	0
KS18_3	898	107	74	85	0	0
KS19_1	926	51	84	121	0	0
KS19_2	988	138	85	111	0	0
KS19_3	987	160	75	129	0	0
KS20_1	864	84	53	67	0	0
KS20_2	978	155	102	121	0	1
KS20_3	887	85	83	72	0	0
KS21_1	1,023	178	88	133	0	1
KS21_2	990	163	90	161	0	1
KS21_3	909	90	103	59	0	0
KS22_1	1,045	185	65	177	0	1
KS22_2	937	106	115	104	0	0
KS22_3	854	67	78	61	0	0
KS24_1	992	178	103	181	0	0
KS24_2	811	47	31	43	0	0
KS25_1	792	44	49	28	0	0
KS25_2	698	23	60	16	0	0
KS25_3	725	37	45	112	0	0
KS26_1	806	41	52	34	0	0
KS26_2	761	33	35	30	0	0
KS26_3	793	30	64	26	0	0
KS27_1	734	30	40	24	0	0
KS27_2	708	23	47	17	0	0
KS27_3	744	24	45	28	0	0
KS28_1	723	23	39	28	0	0
KS28_2	824	31	51	20	0	0
KS28_3	724	25	39	25	0	0
KS29_1	758	19	43	21	0	0
KS29_2	757	17	39	23	0	0
KS29_3	722	26	47	34	0	0
KS30_1	768	38	27	28	0	0
KS30_2	813	53	49	40	0	0
KS31_1	757	15	67	16	0	0
KS31_2	817	25	64	31	0	0
Average	882	121	67	91	0	1
Max	1,054	324	120	230	0	19
Min	671	14	27	14	0	0

Table 6

siRNA																	
Husker administration		30 days		90 days		6 months		9 months		11 months		1 year		14 months		1.5 year	
KS No.	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	
1	0	0	0	0	0												
3	0	1	1	1	0												
4	0	0	0	0	0	1											
5	1	0	0	20	0	1				0	21			0	0	0	
6	1	1															
7	0	0															
8	0	1	1	1	0		1	0				0	1			0	
9	0	0	0	1	0											0	
10	0	0	0	0	0			1	0					0	1	0	
11	0	2	1	1	0												
12	0	0	1	0	1												
13	0	3	0	0	0												
14	0	0	0	0	0												
15	0	1	1	1	0												
16	0	0	0	1	0												
17	0	0	0	0	0												
18	0	0	0	0	0												
19	0	0	0	0	0												
20	1	0	0	0	1												
21	0	0	0	0	1					0	0	0	0	0	0		
22	0	1	0	0	0												
24	0	0															
25	0	0	0	0	0												
26	0	0	0	0	0												
27	0	0	0	0	0												
28	0	0	0	0	0												
29	0	0	0	0	0												
30	0	0															
31	0	0															

miRNA																	
Husker administration		30 days		90 days		6 months		9 months		11 months		1 year		14 months		1.5 year	
KS No.	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	
1	22	126	51	50													
3	54	68	253	23													
4	18	142	135	196													
5	51	128	118	184						113	233			28	446	302	
6	42	28															
7	113	45															
8	193	232	49	549	602	28						18	302			33	
9	79	74	38	552												389	
10	55	204	52	512			338	60						86	243	13	
11	52	327	82	473												55	
12	38	5	464	62													
13	40	95	400	33													
14	128	5	396	50													
15	458	44	28	57													
16	368	63	68	25													
17	131	175	17	129													
18	379	25	2	6													
19	333	131	85	19													
20	320	28	53	331													
21	27	59	37	267						115	236	27	130	55	20		
22	41	246	34	153													
24	46	439															
25	27	130	55	20													
26	19	70	74	27													
27	41	37	20	20													
28	86	11	14	98													
29	17	9	29	10													
30	55	8															
31	90	18															

piRNA																	
Husker administration		30 days		90 days		6 months		9 months		11 months		1 year		14 months		1.5 year	
KS No.	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	
1	16	19	47	26													
3	27	15	65	9													
4	8	46	39	54													
5	34	73	62	85						54	68			11	129	64	
6	21	35														2	
7	21	18															
8	63	76	6	129	156	1						11	92			94	
9	18	65	19	135													
10	21	62	9	115			50	14						28	53	7	
11	34	128	28	126												12	
12	6	2	119	4													
13	31	30	122	27													
14	30	0	126	12													
15	105	6	29	22													
16	93	10	54	11													
17	38	48	4	47													
18	110	1	9	40													
19	85	90	43	16													
20	84	21	27	96													
21	43	33	10	105						25	52	6	18	100	5		
22	43	104	8	66													
24	18	171															
25	6	18	100	5													
26	8	15	8	11													
27	6	10	11	2													
28	7	12	11	9													
29	8	5	8	4													
30	17	2															
31	24	2															

Fig. 1a

Fig. 1b

Fig. 2-1

Patient No. 5

No. 6

Patient No. 7

Huaier ad.
1 month

Patient No. 8

Huaier ad. 1 month 3 months 6 months 9 months 14 months

Fig. 2-2

Fig. 2-3

Fig.3a-1

21/25

30 days

Fig.3a-2

8

31

26

Prostate hyperplasia

15

5,8

12

5,8,10, 21/25

12

5,8,10,
21/25

NFκB

12

NFκB

Fig. 3c-1

Fig. 3c-2

No. 7 Stage IV cancer 30 days 90 days

No.20 Benign adenoma (2.0 x 2.0 cm diameter, no treatment, or Huaier administration)

Fig. 3c-3

13,14

19,20

Fig. 4a

a

30 days

90 days

Fig. 4b

Fig. 5

Huaier ad. 30 days

90 days

Fig. 6

Patient No.5

Patient No.10

Patient No.21/25

[illegible]

Extended Table 2

Patient	Sampling time after Huaier administration									
No.	before	30 days	90days	6 months	9 months	11 months	12 months	14 months	18 months	
1	23.06	20.05	21.09							
3	22.99	14.65	19.6							
4	22.74	21.04	23.77							
5	21.45	19.9	25.54			28.45		17.14	23.48	
6	27.4	22.58								
7	18.51	20.83								
8	29.63	18.34	10.74	29.02	31.66			33.18		
9	23.16	26.02	12.38							
10	31.57	23.27	8.98		41.44			40.88	32.9	
11	24.17	21.2	8.68							
12	8.47	12.03	23.35							
13	7.58	9.44	24.26							
14	13.07	11.84	29.77							
15	10.7	30.66	25.81							
16	11.97	27.01	22.99							
17	34.31	28.1	23.56							
18	14.88	29.76	26.83							
19	11.05	26.31	24.84							
20	30.67	35.74	48.99							
21	25.44	22.13	44.74			26.53*	20.69*	22.14*		
22	20.32	36.91	34.64							
24	34.26	23.15								
25	26.53*	20.69*	22.14*							
26	22.49	15.75	19.92							
27	21.7	19.15	21.75							
28	29.78	28.25	32.33							
29	26.28	24	23.43							
30	22.47	19.36								
31	20.77	22.99								

*: the same

Patient	Sampling time after Huaier administration									
No.	before	30 days	90days	6 months	9 months	11 months	12 months	14 months	18 months	
1	50.04	45.41	53							
3	80.16	35.28	63.57							
4	54.79	34.88	39.16							
5	54.12	50.67	55.49			9.21		34.18	45.4	
6	57.97	56.38								
7	37	46.12								
8	67.05	33.18	35.83	64.21	8.55			39.79		
9	58.04	68.18	46.61							
10	55.78	36.03	39.44		12.52			47.62	36.65	
11	58.89	48.58	39.43							
12	36.52	49.91	50							
13	47.2	42.85	52.84							
14	39.14	43.04	43.93							
15	52.74	64.62	55.82							
16	39.7	53.61	58.45							
17	45.09	46.08	46.77							
18	47.37	59.43	60.89							
19	41.59	51.46	57.81							
20	59.34	68.57	13.88							
21	49.47	53.41	18.36			41.31*	31.46*	38.62*		
22	65.44	11.82	12.02							
24	13.14	49.17								
25	41.31*	31.46*	38.62*							
26	40.07	26.23	33.86							
27	40.55	29.7	33.8							
28	44.58	45.79	47.44							
29	33.51	34.68	33.48							
30	34.88	36.39								
31	30.37	44.66								

*: the same

Extended Table 3

a

iPS-related genes	iPS genes\KS No.	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	24	25	26	27	28	29	30	31
KIT	3,815	Up	*	*	*	*	*	*	*	Up	*	*	*	*	*	*	*	Up	*	*	*	*	Down	Up	*	Down	*	*	*	*
MYC	4,609	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Up	*	*	*	*	*	Down	Up	*	Down	*	*	*	*	*
OCT3/4	5,460	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Up	*	Up	Up	*	*	*	*	*	*	*	*	Down	*	*
SOX2	6,657	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
LIN28A	79,729	*	*	Down	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Down	*	*	*	*
NANOG	79,923	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

b

iPS-related genes	iPS genes\KS No.	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	24	25	26	27	28	29	
KIT	3,815	*	*	*	*	*	*	*	*	*	*	*	*	Up	Up	*	*	*	*	*	*	*	*	*	*	*	*	*	*
MYC	4,609	*	*	*	*	*	Down	*	Down	Down	Up	Up	*	*	*	*	*	*	Down	Down	Down	Down	*	*	*	*	*	*	
OCT3/4	5,460	*	*	*	Up	*	*	*	*	*	*	Up	Up	Up	*	Up	*	Up	*	Down	Down	*	*	*	*	*	*	*	
SOX2	6,657	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
LIN28A	79,729	*	*	Down	*	*	*	*	*	*	*	*	Down	Down	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
NANOG	79,923	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Extended Table 4

sRNA	Type	Average	S.D.	Max.	Mini.
miRNA	mature	87.05	4.15	94.44	71.24
	precursor	6.35	1.72	10.33	3.66
genome	intron	1.33	0.92	3.68	0.21
	intergenic	1.07	1.16	6.39	0.06
	exon	0.51	0.39	1.40	0.06
	repeat	0.46	0.37	1.70	0.05
sncRNA	piRNA	0.06	0.06	0.26	0.00
	rRNA	0.05	0.04	0.19	0.00
	snoRNA	0.03	0.02	0.10	0.00
	tRNA	0.01	0.01	0.03	0.00
	snRNA	0.00	0.00	0.00	0.00
	hairpin	0.00	0.00	0.00	0.00
unknown	unmap	1.94	1.73	12.37	0.30
	Rfam other sncRNA	1.14	1.14	4.94	0.07

Figures

Figure 1

SNP variance after Huaier administration. a. classifications by SNP variation types; b. classifications by splice event types.

Figure 2

Comparison of the numbers of differently expressed genes (DEGs) in each patient by time course of Huaier administration. The asterisks * indicate the time of opportunistic virus infection.

Figure 3 is too large to display. This figure has been added to the Supplementary files section.

Figure 3

Pathway classification and functional enrichment of DEGs. a. Pathway functional enrichment results for up/down regulation genes. X axis represents the terms of pathway. Y axis represents the number of up/down regulation genes. b. The detailed analysis of the Huaier effects on each cancer by KEGG biological pathways²⁵. The pathways responsible for cancer progression, stress reduction, and major immunological responses related to NFkB and TGFb signalling pathway were specifically compared among patients with recovery and poor prognosis. c. Functional enrichment of the factors classified by KEGG pathways. The colour indicates the q-value (high: white, low: blue), the lower q-value indicates the more significant enrichment. Point size indicates DEG number (the bigger dots refer to larger amount). Rich Factor refers to the value of enrichment factor, which is the quotient of foreground value (the name of DEGs) and background value (total gene amount). The larger the value, the more significant enrichment.

Fig. 4a

Fig. 4b

Figure 4

TF-DEG network in each patient by the time course of Huaier administration. a. TF families classified by the encoding DEGs, with each number of up/down regulation. b. TF-DEG predicted network. The red and green dots represent the up-regulated and down-regulated DEGs, respectively. Purple ball represents transcription factor, the greater the node the more DEGs the transcription factor regulate.

Figure 5

Comparison of the numbers of DESs until 90 days of Huaier administration. Upper: Statistics of siRNA, middle: miRNA, lower: piRNA.

Figure 6

Comparison of the numbers of DESs in each patient by time course of Huaier administration.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Figure3.pdf](#)