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Original Article

Stage specific transcriptome analysis of liver tissue from a crossbred Korean Native Pig (KNP × Yorkshire)

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Korean Native Pig (KNP) has a uniform black coat color, excellent meat quality, white colored fat, solid fat structure and good marbling. However, its growth performance is low, while the western origin Yorkshire pig has high growth performance. To take advantage of the unique performance of the two pig breeds, we raised crossbreeds (KNP × Yorkshire to make use of the heterotic effect. We then analyzed the liver transcriptome as it plays an important role in fat metabolism. We sampled at two stages: 10 weeks and at 26 weeks. The stages were chosen to correspond to the change in feeding system. A total of 16 pigs (8 from each stage) were sampled and RNA sequencing was performed. The reads were mapped to the reference genome and differential expression analysis was performed with edgeR package. A total of 324 genes were found to be significantly differentially expressed (|log2FC| > 1 & q < 0.01), out of which 180 genes were up-regulated and 144 genes were down-regulated. Principal Component Analysis (PCA) showed that the samples clustered according to stages. Functional annotation of significant DEGs (differentially expressed genes) showed that GO terms such as DNA replication, cell division, protein phosphorylation, regulation of signal transduction by p53 class mediator, ribosome, focal adhesion, DNA helicase activity, protein kinase activity etc. were enriched. KEGG pathway analysis showed that the DEGs functioned in cell cycle, Ras signaling pathway, p53 signaling pathway, MAPK signaling pathway etc. Twenty-nine transcripts were also part of the DEGs, these were predominantly Cys2His2-like fold group (C2H2) family of zinc fingers. A protein-protein interaction (PPI) network analysis showed that there were three highly interconnected clusters, suggesting an enrichment of genes with similar biological function. This study presents the first report of liver tissue specific gene regulation in a cross-bred Korean pig.

Key words: Korean Native Pig, Yorkshire Pig, Principal Component Analysis, edgeR, MAPK signaling pathway

Introduction

Korean Native Pig (KNP) which has a uniform black coat color and excellent meat quality is well known for full filling consumer's demand for flavored pork meat [1, 2]. KNP produces white colored fat, solid fat structure and good marbling, however, its growth performance is low, while the western origin Yorkshire pig has high growth performance [3]. So a crossbred pig which could be high performing as well as high quality meat producing, was generated (KNP × Yorkshire) [4]. Liver is known to play a significant role in fat and lipid metabolism, although, meat quality of the pig depends upon multiple factors like intramuscular fat content, muscular pH, cholesterol level, drip loss, water holding capacity, texture, breed, feeding style, slaughtering and cooking loss [3, 5], fat content is the major deciding factor of pork meat quality

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[6, 7].

RNA-seq analysis of liver tissue of swine can be useful to decipher the liver fat metabolism and their significance to improve the meat quality. The pig reference genome Sscrofa10.2 still has missing annotation [8], and recently whole genome sequence using third generation sequencing; long read approach (PacBio); has been initiated for annotation of pig genome [9]. Since, RNA-seq is less prone to mapping artifacts, and can be directly mapped to the available reference genome, it can uncover the association between meat quality and liver fat metabolism. To the best of our knowledge, RNA-seq analysis has not been done so far for liver tissue of cross breed pig. In this study, we used transcriptome sequences from liver tissue of 16 crossbreed pig sample (KNP × Yorkshire), using illumina sequencing technology. Sequencing has been done at two different stages ;10 week and 26 week for comparative analysis. Until the age of 10 weeks, piglets are considered as weaners, and from 10 weeks to slaughtering time (around 26 weeks) pigs are considered as rearing pigs. We considered 10 weeks and 26 weeks, to infer gene expression changes, keeping in mind that during this time period gene expression of liver and fat metabolism will change due to the change in feeding regimen and also due to effects of age. After checking for the quality of the raw sequences we identified differentially expressed genes (DEGs). Gene ontology (GO) and KEGG pathway analysis performed to established the relation between fat metabolism and liver through transcriptome analysis. qRT-PCR was performed to validate the identified DEGs.

Materials and Methods

Ethical statement and pig rearing

A total of 16 crossbred pigs between Korean native and Yorkshire breed were used in this study. The experiment was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC no. NIAS2016-848). Feed and water were supplied during the experiment. These piglets were randomly allocated into two groups (n = 8). The two groups were raised for 10 weeks (stage 1) and 26 weeks (stage 2), respectively. The animals were sacrificed at the end of the growth stage and the liver tissues were collected for RNA-seq analysis. The tissues were dissected into pieces (1 cm^3), and snap frozen in liquid nitrogen and stored at –80℃ until use.

Isolation of RNAs, and RNA sequencing

Total RNAs of all the samples were extracted using TRIzol reagent (Invitrogen, United States USA), and quality checked using NanoDrop (Thermo scientific, United States USA). After performing quality control (QC), the samples were used for library preparation. The sequencing library is prepared by random fragmentation of the cDNA sample, followed by 5' and 3' adapter ligation. Adapter-ligated fragments were then PCR amplified and gel purified. For cluster generation, the library was loaded into a flow cell where fragments were captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment was then amplified into distinct, clonal clusters through bridge amplification. After cluster generation, the samples were sequenced in an Illumina Hiseq 4000 sequencer [10]. The sequencing was performed by Macrogen Inc., Korea. The quality of paired end reads of all 16 samples was checked by FASTQC (v0.11.4) program [11]. Low quality bases and adapter sequences were removed with Trimmomatic software [12]. Only good quality trimmed reads were considered for downstream analysis.

Quality analysis, mapping, and assembly of sequenced RNA reads

The reference genome of pig (sus scrofa) and annotation files were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/genome?term=sus%20scrofa) as GCF_000003025.6_Ssrofa11.1_genomic.fa and indexed with Bowtie2 program (Bowtie2 v2.3.4.1) [13]. Paired end clean reads were mapped against the reference genome with HISAT2 (HIASAT2.1.0) alignment program. HISAT is a sensitive and fast alignment program for NGS reads for DNA as well as RNA [14]. Mapped sequences were taken into consideration for assembly through assembler cufflink (cufflink 2.2.1). Cufflink program was used to assemble the transcripts and find their abundance among the samples, with parameter 'min-frags-per-transfrag = 0' and –library-type' and kept all parameters as default [15]. Cuffmerge, a tool of Cufflink suit was used to merged samples and get the merged gtf file for further downstream analysis.

Identification of differentially expressed genes

Differential gene expression analysis was performed with EdgeR, which uses a negative binomial model for dispersion estimate for calculating biological variation [16]. Genes with an adjusted *p*-value (FDR) lower than 0.001 and a two-fold change was considered as significantly differentially expressed in the pairwise comparison of the samples stage 1 and stage 2. The R package Heatmaps was used to generate the DEG heatmap.

Gene ontology and KEGG pathway

The GO database (http://www.geneontology.org/) was used for functional annotation of DEGs. The q value was

calculated and GO terms with q < 0.05 were considered significantly enriched. The biological pathways and functions were analyzed on the basis of q value through DAVID, and ClusterProfiler. Similarly KEGG pathways were examined by keeping q value < 0.05 (http://www.genome.jp/kegg/).

Validation of differentially expressed genes by qRT-PCR

The quantitative real time PCR was used to validate the top five DE genes by using a reverse transcriptase kit (Takara, Japan). 1 µg of total RNA was used to synthesize as the template for quantitative PCR by the SYBR Premix Ex Taq kit (Takara, Japan). Quantitative PCR analyses were conducted using the CFX96 Touch real-time PCR system (Bio-Rad, CA, USA). The PCR conditions were as follows; reverse transcriptase at 60℃ for 90 minutes, followed by denaturing at 95℃ for 8 min and then 40 cycles of replication 94℃ for 15 s and 60℃ for 1 min; then melting curve was run from 65–95℃ at each amplification cycle for the three replicates.

Results and Discussions

Analysis of sequencing reads

Raw reads obtained from transcriptome sequencing, were processed using FastQC and trimmed by removing adaptor sequences with Trimmomatic software [11, 12]. Clean transcriptome sequences were retrieved by removing adapter's sequences, and removing the low quality reads (Phred quality score ≤ 10). The percentage of GC, AT, Q20, Q30 of the cleaned reads were calculated and

Table 2. Read depth and alignment rate for each sample library

Sample	Total reads	Alignment rate(%)
Y5-9A-R	36,089,798	95.43
Y5-3A-R	57,832,074	95.36
K4-4A-R	30,840,079	89.47
Y5-2A-R	32,017,772	93.54
K4-10A-R	31,041,478	96.39
Y5-4A-R	32,144,765	90.89
Y5-6A-R	31,670,261	95.37
Y5-12A-R	31,857,195	91.96
Y5-11A-R	57,832,074	95.48
K4-5A-R	41,949,104	94.52
Y5-10A-R	49,594,201	95.97
K4-8A-R	34,874,734	95.34
Y5-5A-R	38,893,078	95.84
Y5-1A-R	44,952,188	94.98
Y5-7A-R	36,089,798	95.43
Y5-8A-R	57,832,074	95.36

Table 1. Sample wise quality analysis summary

Sample	Total bases	Read count	GC (%)	AT (%)	Q20 (%)	Q30 (%)
Y5-9A-R	8,935,438,084	88,469,684	48.06	51.94	97.43	93.35
Y5-3A-R	7,100,708,848	70,304,048	49.36	50.64	96.98	92.21
K4-4A-R	11,771,586,764	116,550,364	48.47	51.53	97.59	93.71
Y5-2A-R	10,086,340,558	99,864,758	49.07	50.93	97.78	94.08
K4-10A-R	8,535,441,724	84,509,324	49.70	50.30	97.71	93.94
Y5-4A-R	7,915,781,676	78,374,076	48.50	51.50	97.57	93.65
Y5-6A-R	9,133,304,962	90,428,762	48.17	51.84	97.24	92.94
Y5-12A-R	7,743,070,262	76,664,062	49.38	50.62	97.20	92.72
Y5-11A-R	6,505,860,258	64,414,458	50.32	49.68	96.82	92.36
K4-5A-R	7,336,759,382	72,641,182	53.24	46.76	97.26	92.78
Y5-10A-R	6,443,114,614	63,793,214	48.76	51.24	97.52	93.53
K4-8A-R	6,613,583,020	65,481,020	48.68	51.32	97.42	93.28
Y5-5A-R	6,532,243,074	64,675,674	53.04	46.96	96.74	92.02
Y5-1A-R	6,311,284,364	62,487,964	50.86	49.14	96.52	91.86
Y5-7A-R	6,323,473,852	62,608,652	50.23	49.77	97.70	93.78
Y5-8A-R	6,565,794,870	65,007,870	50.86	49.14	96.21	91.11

shown in Table 1 for all the sixteen samples.

Clean reads of all samples were considered for alignment with the reference genome of pig (GCF_000003025.6_Ssrofa11.1_genomic.fna). HISAT2 was used for alignment, alignment percentage of all samples are between 89 % and 95 % as shown in Table 2. The aligned samples were counted using featurecounts (ver 1.6.3) in the Subread package [17].

Differentially expressed gene analysis

Principal Component Analysis (PCA) showed that the samples clustered according to stages (Fig. 1A) and Volcano plot were generated to show the DEGs between stage 1 and stage 2 as shown in Fig. 1B. A total of 20,396 clean mapped transcripts were considered for further analysis, and a total of 324 genes were found to be significantly differentially expressed ($|log2FC| > 1$ & $q < 0.01$), out of which 180 genes were up-regulated and 144 genes were down-regulated. Hierarchical clustering of the DEGs showed that the samples clustered based on condition (stage 1 vs stage 2) in Fig. 2. In Table 3, we report DEG that plays an important role in fat and lipid metabolism for example PATZ1 gene is involved in synthesis of long-chain polyunsaturated fatty acids (LU-PUFAs) by participating with the FADS1 (fatty acid desaturase 1) and FADS2 genes [18]. Fatty acids metabolism depends upon competitive binding between the PATZ1 and SP1 (specificity protein 1) and SREBP1c (sterol regulatory element-binding protein 1c), which determines the enhancer activity and regulates the expression of FADS1 [18–20]. Similarly, ZNF202 was found to be upregulated, it acts as a transcriptional receptor that can bind to the regulatory part of multiple genes involved in lipid metabolism. ZNF202 a zinc finger protein was reported to play a role in lipid metabolism in humans [21]. Another upregulated gene HAND2 (heart and neural crest derivatives expressed 2) is significantly associated with adipose differentiation through NOTCH signaling pathway, which is involved in lipid metabolism [22].

TCF4 is also responsible for lipid metabolism to reduce the fat deposition in the human body, and can interlinked with pig lipid metabolism [23]. It has been reported that the NHLH1 gene is also associated with lipid metabolism as well as neurogenesis [24]. The downregulated ZIC3 is

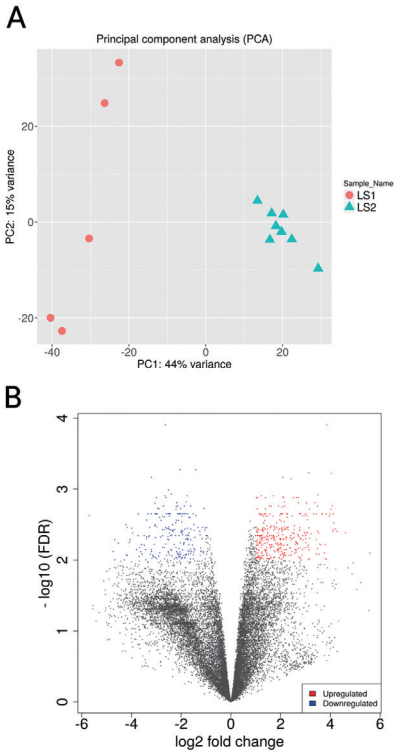

Fig. 1. Comparative analysis of transcriptome profiles of porcine liver from 10 and 26 weeks. (A) Principal component analysis (PCA) of transcripts at stage1 (10 weeks) and stage 2 (26 weeks) (B) Volcano plot showing the threshold applied for identifying significant differentially expressed genes (DEGs). PCA, principal component analysis; DEGs, differentially expressed genes.

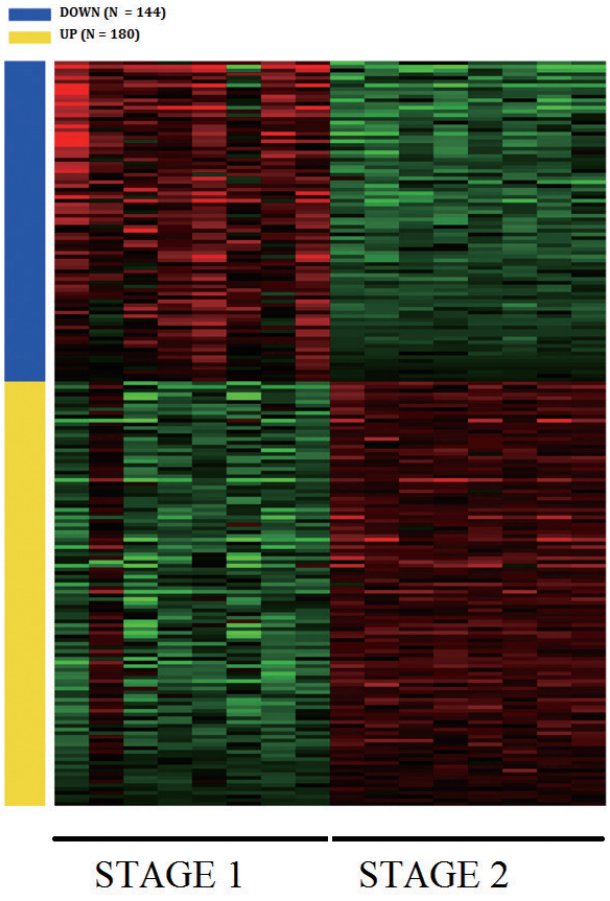

Fig. 2. Heatmap of differentially expressed genes based on normalized gene count data showing the change in expression between Stage 1 and Stage 2.

Table 3. List of differentially expressed genes

Expression	Motif	TF	TF family	FDR
Down regulated	GGGG	ZNF202	C2H2 ZF	0.00E+00
	GGGGGTGG	ZNF281	C2H2 ZF	0.00E+00
	GGGGGGT	ZIC5	C2H2 ZF	0.00E+00
	GGGGGGGGGG	SP1	C2H2 ZF	0.00E+00
	TGTCAGGGGGC	INSM1	C2H2 ZF	0.00E+00
	GGGGCCCAAGGGGG	PLAG1	C2H2 ZF	0.00E+00
	GGAGGAGGAGGGGGAGG	ZNF263	C2H2 ZF	0.00E+00
	GGGCGGGGC	KLF5	C2H2 ZF	0.00E+00
	GGGGGGGGGCC	PATZ1	C2H2 ZF	0.00E+00
	GGGTGGTC	ZIC3	C2H2 ZF	0.00E+00
	CACAGCGGGGGGTC	ZIC4	C2H2 ZF	0.00E+00
	CGGGGGGCCC	ENSSSCG00000004127	C2H2 ZF	0.00E+00
	TGCCCTGAGGC	TFAP2A	AP-2	3.50E-15
	GGGTGGGGC	KLF4	C2H2 ZF	6.50E-15
	CTTCGTGGGGGGTCT	GLIS1	C2H2 ZF	3.00E-14
	GGGTGGTC	ZIC2	C2H2 ZF	4.50E-14
	CCACCTG	ATOH8	bHLH	5.30E-14
	GGGTGGTC	ZIC1	C2H2 ZF	8.10E-14
	GCCCCCGAGGC	TFAP2C	AP-2	9.10E-14
	GCGTGGG	ENSSSCG00000014333	C2H2 ZF	2.90E-13
Up regulated	GGGG	ZNF202	C2H2 ZF	6.60E-02
	AATGCCAGACGC	HAND2	bHLH	6.60E-02
	GGGGGGGGGCC	PATZ1	C2H2 ZF	6.60E-02
	CAGGTG	TCF4	bHLH	6.60E-02
	CCACCTG	ATOH8	bHLH	6.60E-02
	CAGGTG	TCF12	bHLH	6.60E-02
	CAGGTG	MESP1	bHLH	6.60E-02
	GGGCGTG	KLF12	C2H2 ZF	7.50E-02
	CGCAGCTGCG	NHLH1	bHLH	2.30E-01

involved in activation of zinc finger transcription factor during beginning of gastrulation [25]. Some other down-regulated gene like PLAG1, that can affect the male fertility in cattle and in several species [26].

Pathway analysis

GO consists of three main components like, biological process, molecular function, and cellular component. In this study, our findings suggesting that the DE genes of porcine's liver tissue of two stages are involved in fat and lipid metabolism. Gene enrichment analysis (at $q < 0.05$) of DE genes showed enrichment of multiple GO terms. We have used q value, since we have taken our gene list and compared with every single pathway that is annotated in DAVID. Genes involved in biological process (Fig. 3A) such as cell division, DNA replication, regulation of signal transduction by p53 class member, translational initiation, G1/S transition of mitotic cell cycle, nucleotide-excision repair, DNA gap filling, positive regulation of telomerase activity, protein phosphorylation, activation of GTPase activity, cellular components like kinetochore, ribosome, focal adhesion etc. and performing molecular function such as structural constituent of ribosome, Poly(A) RNA binding, DNA helicase activity, microtubule motor activity etc. were found to be enriched.

We then investigated the significantly enriched KEGG pathways ($q < 0.05$) and found pathways like cell cycle, P13K-Akt signaling pathway, Gap junction, Ras signaling pathway, Phagosome, p53 signaling pathway, MAPK signaling pathway, FoxO signaling pathway etc. to be significantly enriched (Fig. 3B).

Protein-protein interaction analysis

We then performed, protein-protein interaction (PPI) network analysis with the significant DEGs identified in this study using STRING database and considered the nodes as genes and the edges are the interaction between the nodes. Three clusters involving genes that functions in cell cycle, DNA replication, and DNA repair, Fanconi Anemia pathway were found to have significant interactions, as shown in Fig. 4.

qRT-PCR analysis to validate the top five DEG genes

We have selected the top 5 DEGs like PKP1, SLC17A3, OR10P1, LRFN4, IL2 which are involved in fat metabolism of liver tissues of crossbred pig. The expression levels of five genes were analyzed in stage 1 vs. stage 2. As shown in Fig. 5A, qRT-PCR is also corroborating the findings of RNA-seq findings. The Fig. 5B is showing the correlation (r^2) at value 0.9428 and ΔCT and LOG2FC of RNA-seq analysis. Many similar interpretations have been reported earlier for validation of DE genes through qRT-PCR [27–30].

Conclusion

This study presents the first report of liver tissues gene regulation in a cross-bred Korean pig. A detailed and comprehensive investigation of mRNAs in stage specific liver tissue may serve as a good resource for further study. By analyzing the stage specific RNA-Seq data of pig liver tissues, we showed the expression profile of mRNAs and their association with fat metabolism. Through GO, pathway and PPI analysis we found genes and TFs that are involved in fat metabolism, cell cycle, DNA repair and replication and signal transduction to be significantly enriched in stage 2 pigs relative to stage 1. However, functional genetics experiments are still need-

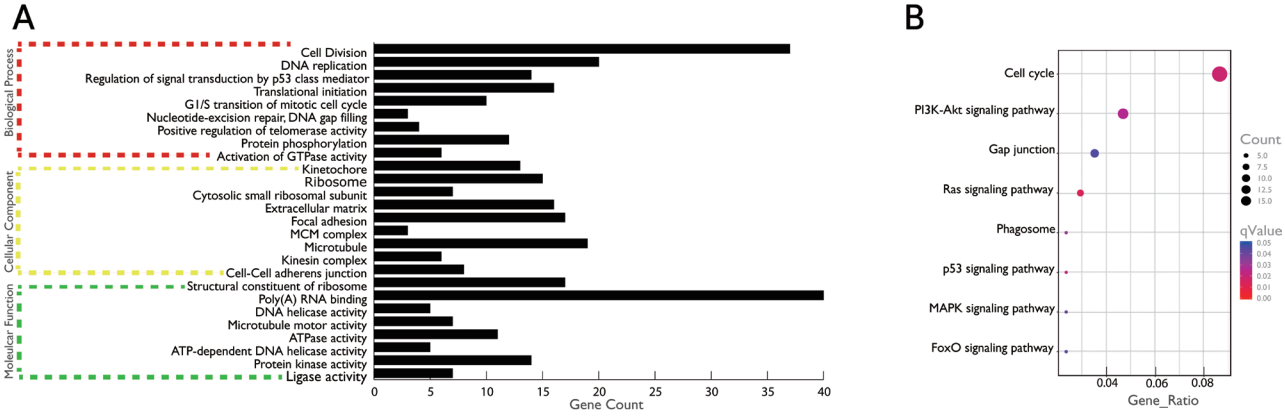

Fig. 3. Functional annotation analysis of DEGs, (A) Gene ontology enrichment analysis of DEGs classified under biological process, cellular component and molecular functions. Only GO's found to be significant a $q < 0.05$ was plotted. (B) KEGG pathways enriched amongst the identified DEGs ($q < 0.05$). DEGs, differentially expressed genes.

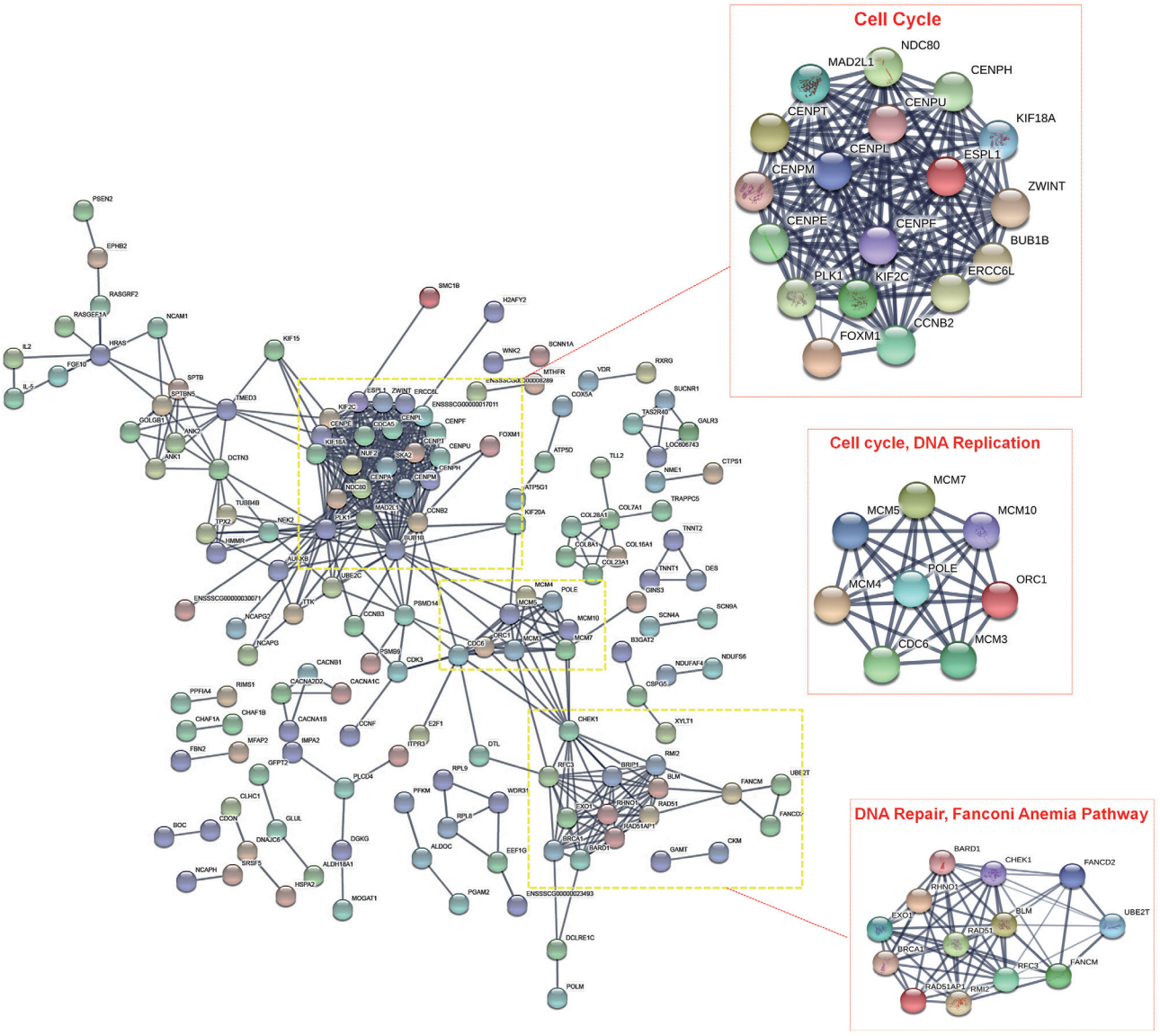

Fig. 4. Protein-protein interaction network analysis of DEGs. Three highly connected clusters like cell cycle, DNA replication, and DNA repair. DEGs, differentially expressed genes.

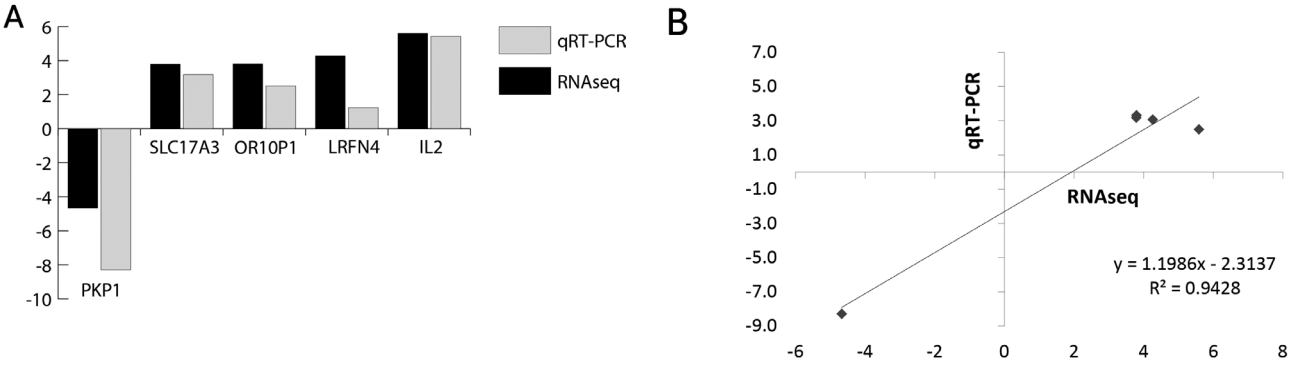

Fig. 5. (A) Expression levels of selected genes from RNAseq analysis (black bar) and their validation by qRT-PCR (grey bar). (B) R^2 is the correlation of expression levels between the two methods.

ed to validate the functional association of mRNAs presented in this study.

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