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Effects of Different Molecular Weight Hyaluronic Acid on the Transcriptome of Inflammation-related Colon Cancer Cells

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Abstract:
In order to study the main signaling pathways and the expression rules of key genes differentially expressed by different molecular weight hyaluronic acid on human colon cancer cells, Chinese hamster ovary cells (CHO) and pMH3 were used in this study to express the chimeric hyaluronidase PH20, and obtain stable expression and expression. Hyaluronic acid fragment HA35 was prepared by enzymatic digestion with high-purity PH20. Human peripheral blood leukocytes and human colon cancer cell line (HT-29) were used to establish inflammation-related colon cancer cell models, and they were randomly divided into control group and experimental group. The control group was cultured with complete medium for 24 h, in the experimental group, 20 μg/mL of high-molecular-weight hyaluronic acid (HA) and complete medium of HA35 were added for 24 h, and the effect of hyaluronic acid on the expression of TNF-α in inflammation-related colon cancer cells was detected by enzyme-linked immunosorbent assay (ELISA). Human colon cancer cells were collected and total RNA was extracted, reverse-transcribed to establish a cDNA library, and high-throughput sequencing was performed using an Illumina Hiseq X Ten sequencer to establish a transcriptome analysis platform at the mRNA level of HT-29 cells.Transcriptome analysis showed that there were 1372 differentially expressed genes in the HA treatment group compared with the control group, and 966 differentially expressed genes in the HA35 treatment group compared with the control group. These differential genes were mainly related to Rap1, Ras, PI3K-Akt, TGF-β and other inflammatory signaling pathways are related, and differential expression analysis found that HA may inhibit the further development of colon cancer cells by reducing the expression of inflammatory factors such as TNF, IL-17, and IL-22. This study can provide a reference for in-depth exploration of the molecular response mechanism of hyaluronic acid to inflammatory changes in colon cancer cells.

Keywords: HA, HT-29, Anti-inflammatory function, Transcriptome analysis.

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I. INTRODUCTION

Colon cancer is one of the most common malignant gastrointestinal tumors in the world. It is estimated that there are more than 600000 deaths due to colon cancer every year in the world[1], and more than 190000 deaths due to colon cancer in my country each year[2]. The occurrence of colon cancer is a complex multi-factorial process, and the colon tissue of patients often has high expression of inflammatory factors such as IL-1, IL-6, TNF-α and TGF-β. mitosis and other related signaling pathways, and then promote the occurrence and development of inflammation-related tumors[3].

As a naturally occurring glycosaminoglycan, hyaluronic acid is widely present in bacteria and eukaryotes and is one of the main components of the extracellular matrix, which is composed of repeating N-acetylglucosamine and D-glucuronic acid through glucose The uronic acid $\beta(1\rightarrow3)$ bond and the hexosamineβ $(1\rightarrow4)$ bond are continuously connected[4]. The half-life of hyaluronic acid in the human body is 15 hours, and about 1/3 of the hyaluronic acid in the human body undergoes the renewal process of degradation and resynthesis every day, that is, a state in which hyaluronic acid and hyaluronic acid fragments coexist[1]. The decomposition of hyaluronic acid depends on hyaluronidase (Hyaluronidase, HAase). So far, six HAases have been identified in the human body, namely hyaluronidase 1-4, PH20, HYAL-1. Hyaluronic acid participates in related signaling pathways by binding to cell surface receptors to regulate the expression of cellular inflammatory factors. Studies have found that high molecular weight hyaluronic acid interacts with HYAL1 receptors on the surface of U-CH1-N cells (a notochordal nucleus cell line). When combined, the expression of TNF-α may be inhibited by regulating the p38 and Erk1/2 pathways, confirming the efficacy of hyaluronic acid on intervertebral disc (IVD) inflammation[4].

In this study, by establishing an inflammation-related colon cancer cell model, treating inflammation-related colon cancer cells with hyaluronic acid of different molecular weights, performing colon cancer cell transcriptome sequencing, constructing a transcriptome database, identifying significantly differentially expressed cellular pathways, and comparing inflammation Differences in gene expression between factor changes and control groups, and functional annotation to reveal the transcriptional profiling characteristics of the molecular response mechanism of inflammatory colon cancer cells to changes in the molecular weight of hyaluronic acid, in order to further reveal the effect of hyaluronic acid on colon cancer cell inflammation The changed physiological and biochemical regulatory mechanisms provide a reference for future research on hyaluronic acid in the treatment of inflammation-related colon cancer.

II. MATERIALS AND METHODS

Experiment material. Chinese hamster ovary cell CHO and expression plasmid pMH3 were provided by Shaoxing Huihui Biotechnology Co., Ltd.; human colon cancer cell line HT-29 was purchased from Proceeds; hyaluronic acid was purchased from Bloomage Biotechnology Co., Ltd.; RPMI-1640 Purchased from Sigma company; hyaluronic acid standard was purchased from Bloomage Biotechnology Co., Ltd.; carbon dioxide incubator was purchased from Sanyo company; high pressure steam sterilizer was purchased from Japan Sanyo company; ; Disposable vacuum

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blood collection tube and human venous blood leukocyte separation kit were purchased from Tianjin HaoyangHuake Biotechnology Co., Ltd.; Human TNF-α enzyme-linked immunosorbent assay detection kit was purchased from R&D system company of the United States; microplate reader was purchased from the United States Bo Teng Instrument Co., Ltd.; fetal bovine serum was purchased from Shanghai Sangon.

Expression and purification of recombinant hyaluronidase. Referring to the human hyaluronidase PH20 gene sequence, Primer premier 5.0 software was used to design upstream and downstream primers, and the gene fragments were amplified by PCR, and the PCR products were recovered by agarose gel analysis. The hyaluronidase nucleotide sequence and the expression vector were digested and recombined, and the recombinant plasmid was transformed into the competent Agrobacterium, and the resistant colonies were screened to extract the recombinant plasmid for digestion and identification. CHO cells and vectors were transiently transfected by electroporation method, and positive clones and high-expression cloned cell lines were screened out and expanded into a 20 L torrent animal cell reactor.

The cell culture medium was collected, centrifuged at 4750 g/min for 8 min in a high-efficiency centrifuge, and filtered with three pore size gradient membranes to remove impurities such as cells and cell debris. The pH of this concentrate was adjusted to 7.0 and filtered again to obtain a clear protein solution.

The MEP-HyperCel mixed-mode chromatography packing from PALL, the EMD SO3-(M) strong cation chromatography packing from Merck and the SP Sepharose F.F strong cation chromatography packing from GE were used to perform hydrophobic chromatography and cation exchange layer on the protein solution in turn. analysis. The eluate was collected and aliquoted into 15 mL sterile centrifuge tubes with a 0.22 μm sterile filter, stored in a -20℃ refrigerator, and sampled for protein concentration and activity detection.

HA solution preparation.An appropriate amount of 1600 kDa injection-grade hyaluronic acid was taken and dissolved in water for injection to obtain 20 mg/mL HA solution.

HA35 solution preparation. Take 2.0 g of 1600 kDa injection-grade hyaluronic acid, dissolve it in water for injection, add sodium chloride (final concentration 80-90 mmol/mL), magnesium ion (final concentration 1 mmol/mL), and reconstitute it in sequence. Human hyaluronidase (final concentration 15 000 U/g), mix well, and make up to 100 mL with water for injection. The reaction was carried out at 37℃ for 10 min, 20 min, 40 min, 1 h, 2 h, 3 h, and 4 h, 5 h, and 6 h, respectively. The molecular weight of the enzymatic hydrolysis product was determined by agarose gel method, and the hyaluronic acid fragments were averaged. The molecular weight reached the expected 35 kDa as the optimal digestion time. Then sodium chloride (35-45 mmol/L) was added to adjust the osmotic pressure to 280-300 mOsm/L, and the residual recombinant human transparent was inactivated by heating at 95℃ for 45 min. 20 mg/mL HA35 solution was obtained by filtration

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through a 0.22 μm membrane filter. The molecular weight of hyaluronic acid was determined by agarose gel electrophoresis.

Determination of anti-inflammatory activity of hyaluronic acid. Take logarithmic growth phase human colon cancer cell line HT-29 cells, resuspend the cells in McCoy's 5A complete medium containing 10% fetal bovine serum and 1% penicillin/streptomycin, and inoculate 1×10^4 cells per well, at 37°C, 5% CO_2 under the condition of adherent culture for 24 h, on the day of the experiment, freshly extracted leukocytes (1×10^5 cells) were added to the 96-well plate of monolayer cultured HT-29 cells to activate the inflammatory response of colon cancer cells. The control group was added with 100 uL of complete medium, and the experimental group was added with 100 μL of HA35 and HA solutions respectively to make the final concentration of 20 μg/mL. After culturing for 24 h, the supernatant was collected, the OD450 absorbance value of different samples was detected by TNF-α detection kit, the standard curve was drawn, and the expression level of TNF-α was calculated. The experiment was repeated three times.

Effects of hyaluronic acid on the transcriptome of inflammation-related colon cancer cells. Take 5×10^5 HT29 cells and inoculate them in a 6-well plate. When the cell density reaches 90%, discard the medium and wash with PBS twice, and add 100 μL of leukocytes with a certain concentration to each well. The control group was added with 100 μL of complete medium, and the experimental group was added with 100 μL of HA35 and HA solutions respectively to make the final concentration of 20 μg/mL. After culturing for 24 h, the cells were washed with PBS, and the cells were stripped with a cell scraper. The cells were collected together with the PBS solution into a 2.0 mL EP tube without nuclease, and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the pelleted cells were collected.

cDNA library preparation and transcriptome sequencing. First, use magnetic composite particles with Oligo(dT) 25 to extract mRNA with polyA tail, then add elution buffer to collect mRNA; use fragmented mRNA as template, add random primers and reverse transcriptase to synthesize a strand of cDNA, and then synthesize double-stranded cDNA; use AMPure XP beads to collect cDNA fragments of suitable length for library construction; amplify and enrich cDNA fragments through PCR reactions with a limited number of cycles. Finally, the concentration and integrity of RNA samples were detected by Qubit® 3.0 and Agilent 2100, respectively. After the RNA samples are qualified, they are used to construct a transcriptome sequencing library, analyze the library sequencing quality value, GC content, etc., and finally obtain the off-machine data Raw Reads. The library construction and sequencing work were entrusted to Harbin Botai Biotechnology Co., Ltd.

Screening and annotation of differentially expressed genes. Clean Reads were aligned to the reference genome using HISAT2. Differentially expressed genes were analyzed using edgeR software [9]. After standardization by TMM (Trimmed Mean of M value), 2-fold difference, ie $|\log2 \text{Fold Change}|>1$, and FDR < 0.05 were selected as the screening criteria for differentially expressed genes. Annotate the differentially expressed genes to the GO[10] and KEGG[11] databases

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respectively, and apply the hypergeometric test method to find the GO term and KEGG pathway that are significantly enriched in the differentially expressed genes compared with the whole genome background, so as to determine the difference between the differentially expressed genes and the KEGG pathway. Which biological functions and pathways are closely related, GO and KEGG enrichment analysis were performed using TopGO and KOBAS, respectively.

Statistical analysis. Prism 8.0 software was used to make graphs, and SPSS 20.0 software was used to analyze t-test. TNF-α expression data were expressed as mean ± standard deviation, and $P< 0.05$ was used as the criterion for significant difference.

III.RESULTS AND DISCUSSION

Expression and activity detection of hyaluronidase.The expanded cultured cell culture medium was pretreated by centrifugation, extracted by Rukawa, and the expression and purification of hyaluronidase were detected by SDS-PAGE and HPLC. The results are shown in Fig1. The molecular weight of hyaluronidase is about 70 KD, and the purity of the target protein detected by HPLC is as high as 98.75%. The results are shown in Fig 2. According to the hyaluronidase activity detection method (DNS method), its activity was determined to be 27 000 U/mL.

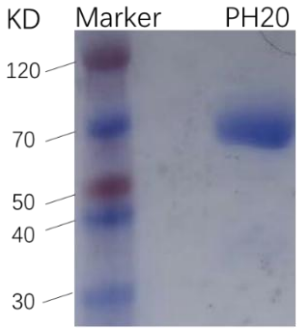

Fig1: Protein SDS-PAGE detection

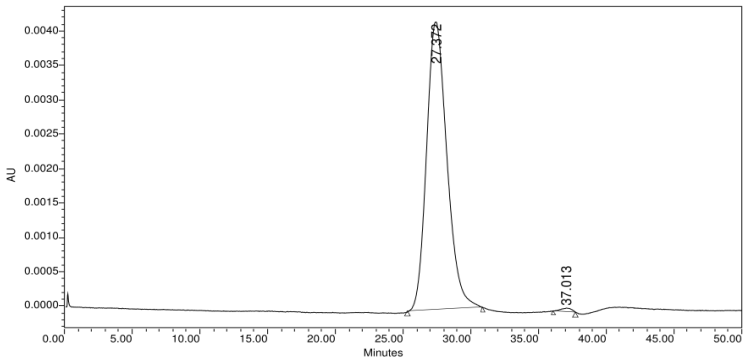

Fig2: Protein HPLC detection

Hyaluronic acid molecular weight. Hyaluronic acid was digested by recombinant human hyaluronidase (PH20) for 10 min (Lane-1), 20 min (Lane-2), 40 min (Lane-3), 1 h (Lane-4), 2 h (The electrophoresis results of hyaluronic acid fragments obtained after Lane-5), 3 h (Lane-6), 4 h

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(Lane-7), 5 h (Lane-8) and 6 h (Lane-9) are shown in Fig 3 , the results showed that the molecular weight of the final product of the enzymatic hydrolysis could reach the expected 35 KDa when the enzymatic hydrolysis time was 2, 3, 4, 5 and 6 h.

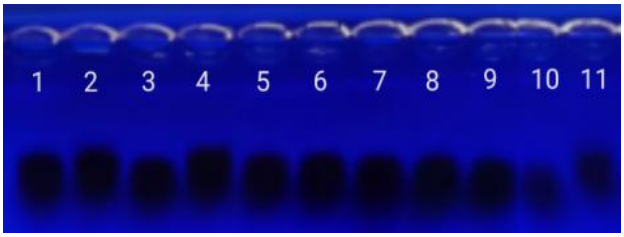

Fig3:Agarose gel electrophoresis detection

Analysis of anti-inflammatory activity. The effect of different molecular weight hyaluronic acid on the expression level of TNF-*α* in human colon cancer cells was detected by TNF-*α* kit. The results are shown in Fig4. Compared with the control group, the change of HA35 concentration had no significant change in the expression level of TNF-*α*, and the expression level of TNF-*α* decreased significantly with the increase of HA concentration in a dose-dependent manner.

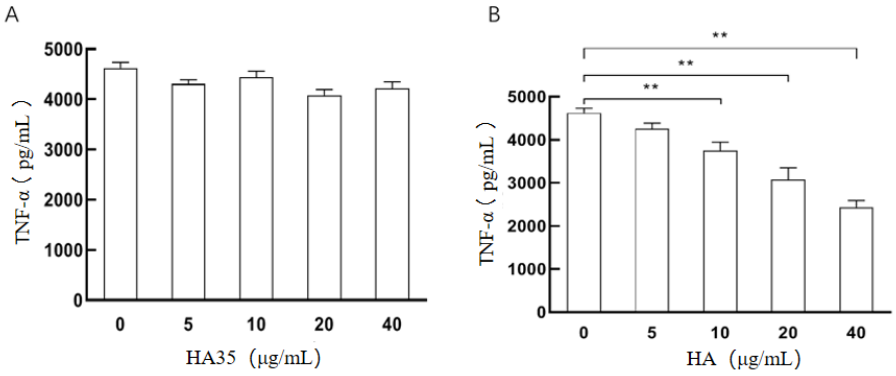

Fig 4:The effect of hyaluronic acid on the expression of TNF-*α* in inflammation-related colon cancer cells

Transcriptome sequencing results statistics. It can be seen from TABLE I that there are 198249254 pure transcriptome reads in the 9 samples, 146379613 unique aligned reads, and 9296140 multiple aligned reads. The percentage of Q30 bases is between 93.00% and 94.26%, and the GC content of each sample after filtering is between 40% and 50%. As can be seen from Fig 5, the FPKM values of the 9 samples spanned 8 orders of magnitude from 10^{-3} to 10^{4}, and there was no significant difference in gene expression between samples (P> 0.05), indicating that the quality of transcriptome sequencing is high and the data is reliable, which can be carried out. Next analysis.

TABLE I. Transcriptome sequencing statistics of colon cancer cells

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Sample	Clean reads	Uniquely reads	Mulitiple reads	GC Content	Q30 Percentage
Control 1	20604755	15210643	971679	48.00%	93.45%
Control 2	22561312	16676408	1057218	48.00%	93.00%
Control 3	21097134	15644056	1023200	48.00%	93.20%
HA35 1	20656497	14984184	947503	48.00%	93.40%
HA35 2	23820983	17284985	1066256	48.00%	93.08%
HA35 3	23166269	16674825	1049966	48.00%	94.05%
HA 1	21654132	15743164	985527	48.00%	93.18%
HA 2	21029210	15133368	921178	48.00%	93.59%
HA 3	23658962	19027980	1273613	48.00%	94.26%
Total	198249254	146379613	9296140		

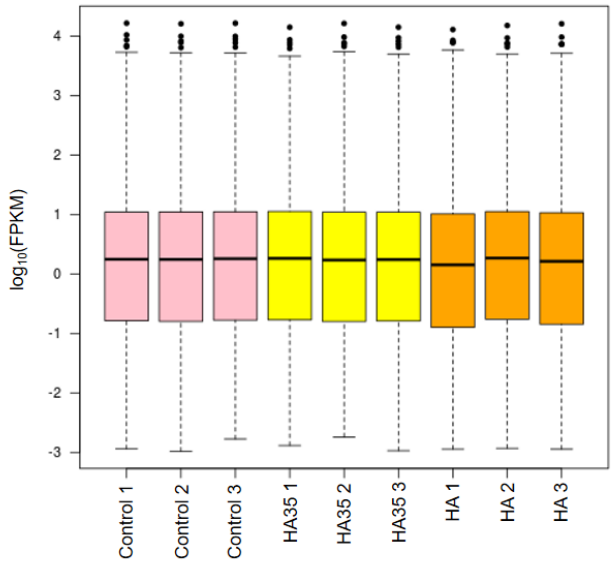

Fig 5: Comparison box plot of FPKM density distribution of each sample

Analysis of differential gene expression in colon cancer cells.The differentially expressed genes located in 9 samples were screened, and a total of 2338 differentially expressed genes were screened. As can be seen from Fig 6, compared with the control group (Control), the HA35 experimental group had a total of 966 differentially expressed genes, 638 up-regulated genes, accounting for 66%, and 328 down-regulated genes, accounting for 34%. There were 1372 differentially expressed genes in the HA experimental group, 1092 up-regulated genes, accounting for 80%, and 280 down-regulated genes, accounting for 20%. Compared with the HA35 experimental group, the HA experimental group produced significantly more differential genes, resulting in significant gene expression changes.

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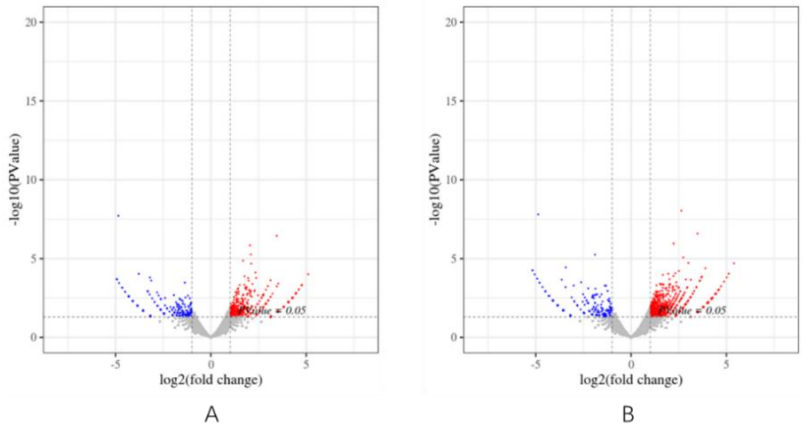

Fig 6:HA35 group(A)and HA group(B)differential gene volcano map

Note: The abscissa represents the fold change of gene expression ($\log_2$ Fold change), the ordinate represents the significance level of the differential gene ($-\log_{10}$ P), red represents up-regulated genes, and green represents down-regulated genes.

GO enrichment analysis of differentially expressed genes.Functional classification and annotation of genes and proteins by GO, classification by Molecular Function (MF), Cellular Component (CC) and Biological Process (BP), classification of differentially expressed genes by GO Annotations include 3 subjects, cellular components, molecular functions, and biological processes. As can be seen from Fig 7, 833 GO entries were significantly enriched in the GO enrichment analysis of the HA35 experimental group, of which 631 were related to biological processes, 61 were related to cellular components, and 141 were related to molecular functions. It can be seen from Fig 8 that 1049 GO entries were significantly enriched in the GO enrichment analysis of the HA experimental group, of which 785 were related to BP, 114 were related to CC, and 150 were related to MF. The number of genes annotated to the same entry was different in different treatments. Compared with HA35, there were more HA-enriched entries, indicating that inflammation-related colon cancer cells may promote molecular functions, cellular components, and biological processes under the action of HA. expression of different functional genes.

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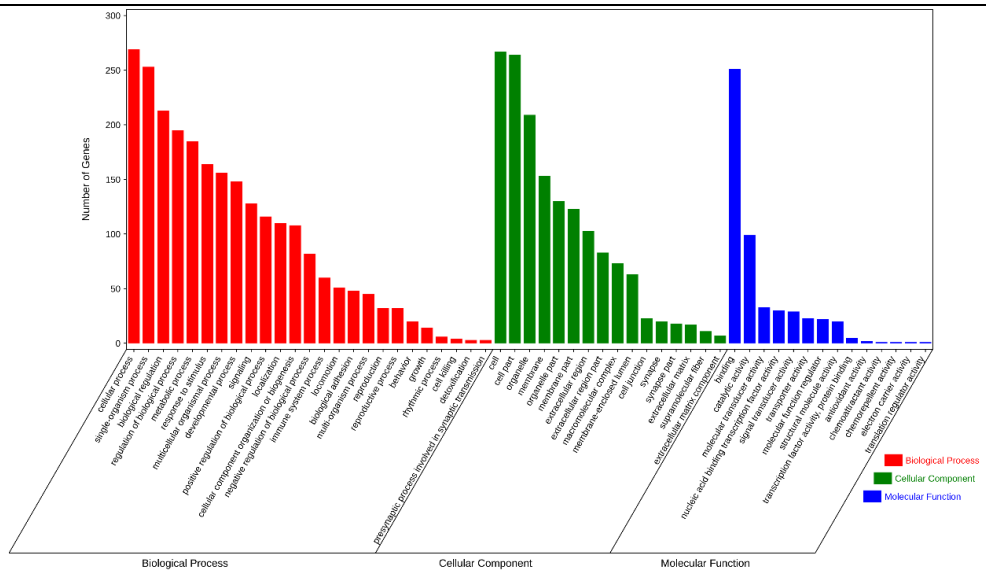

Fig 7: GO classification of differentially expressed genes between HA35 VS Control

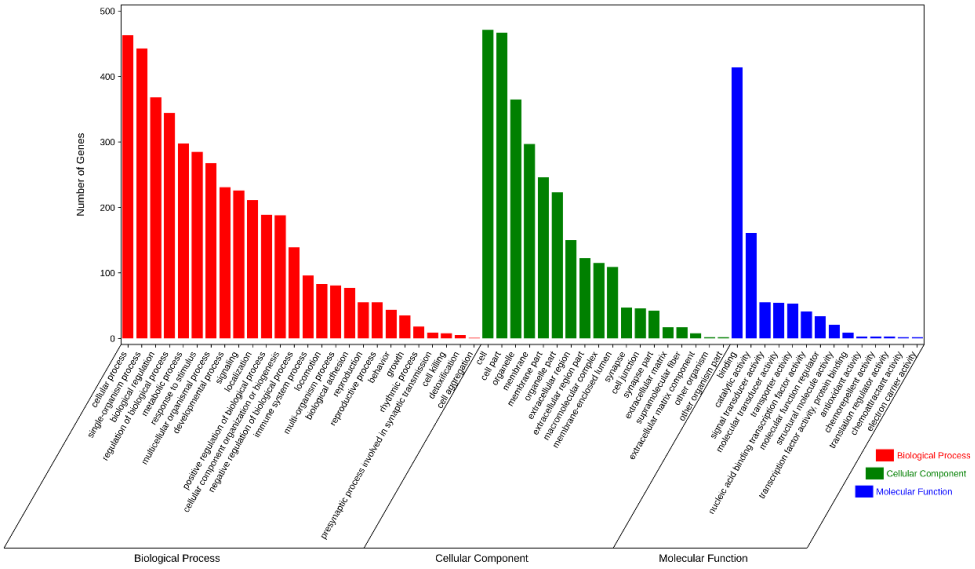

Fig 8:GO classification of differentially expressed genes between HA VS Control

KEGG pathway enrichment analysis of differentially expressed genes.In order to precisely analyze the effect of hyaluronic acid on inflammation in colon cancer cells, the inflammation-related signaling pathways enriched by the differentially expressed gene KEGG were analyzed. As can be seen from TABLE II, a total of 57 samples were enriched in HA35 vs Control, among which TGF-*β* (hsa04350), PI3K-Akt (hsa04151), and cAMP (hsa04024) signaling pathways related to inflammation were significantly enriched, contains 6, 13, and 8 genes, respectively; a total of 66 genes were enriched in HA vs Control, of which 8 genes were significantly related to inflammation, 15 genes were significantly enriched in Rap1 (hsa04015), 14 genes were significantly enriched in

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Ras (hsa04014), and 18 genes were significantly enriched PI3K-Akt (hsa04151), 10 genes were significantly enriched to cGMP-PKG (hsa04022), 7 genes were significantly enriched to TGF-*β* (hsa04350), 9 genes were significantly enriched to Hippo (hsa04390), 14 genes were significantly enriched to MAPK (hsa04010) and 9 genes were significantly enriched to Wnt (hsa04310). It can be seen that HA has a greater impact on the inflammation-related signaling pathways of colon cancer cells, and the impact of high molecular weight hyaluronic acid is more significant. Therefore, HA was used as the target substance for further analysis.

TABLE II. Statistics of differentially expressed genes KEGG enrichment pathway

Treament	Signal pathway	Pathway	Gene number	P value
HA35 vs Control	TGF-*β* signal pathway	hsa04350	6	0.0066
	PI3K-Akt signal pathway	hsa04151	13	0.0091
	cAMP signal pathway	hsa04024	8	0.0331
HA vs Control	Rap1 signal pathway	hsa04015	15	0.0003
	Ras signal pathway	hsa04014	14	0.0025
	PI3K-Akt signal pathway	hsa04151	18	0.0040
	cGMP-PKG signal pathway	hsa04022	10	0.0104
	TGF-*β* signal pathway	hsa04350	7	0.0107
	Hippo signal pathway	hsa04390	9	0.0167
	MAPK signal pathway	hsa04010	14	0.0173
	Wnt signal pathway	hsa04310	9	0.0206

Analysis of related inflammatory factors. To investigate the Effect of high molecular weight hyaluronic acid on inflammatory gene expression in inflammation-related colon cancer cells. From TABLE III, differentially expressed genes such as interleukin 17C (IL17C), interleukin 22 (IL22), tumor necrosis factor (TNF) and interferon 16 (IFNA16) were screened out. The inhibition rate was as high as 59.9%, and the TNF inhibition rate was 40.5%. The gene expression of TNF was consistent with the ELISA results, which further indicated the validity of the transcriptome data.

TABLE III. Analysis of inflammation related differential genes

Gene name	FPKM		Pvalue	
	HA	Control	HA	Control
IL17C	30.499 8	76.000 7	0.000 3	0.000 5
IL22	0.000 0	0.155 4	0.041 1	0.000 0
TNF	0.990 5	1.664 6	0.238 5	0.046 9
IFNA16	0.000 0	0.159 8	0.000 2	0.000 2
CCL8	2.786 5	1.083 8	0.000 0	0.001 4

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IV. CONCLUSION AND DISCUSSION

There is currently no report on the study of hyaluronic acid in inflammation-related colon cancer cells. In this study, a chimeric human hyaluronidase gene expression vector pMH3/PH20 was constructed. The hyaluronidase with high expression and high enzymatic activity was obtained through transfection, expansion culture, purification and other techniques. The enzymatic activity of PH20 was 27000U/mL. It is 1.7 times higher than the PH20 enzyme activity (1000U/mL) cloned by Liu Xia et al[12]. Using the MO2/PH20 expression vector. HMWHA was degraded into small molecular fragments HA35 by PH20 digestion method, and its molecular weight was similar to that of hyaluronic acid fragments detected by GPC-MALLS method by Wang Feng et al[13].

Inflammation of human tissue is mainly caused by the infiltration of leukocytes in the blood into the blood vessels and the release of inflammatory factors (such as TNF-α, etc.) [14]. Previous experiments found that the expression of TNF-α was lower than 100 pg/mLwhen leukocytes or HT29 were cultured alone, and the expression of TNF-α increased sharply to over 4000 pg/mL when leukocytes were co-cultured with HT29, indicating Inflammatory factors released by leukocytes when co-cultured with HT29 cells stimulated the inflammatory response of HT29 cells. Transcriptome differential expression analysis showed that hyaluronic acid had a significant effect on the HT29 signaling pathway and the expression of inflammatory factors, mainly involving TGF-β, PI3K-Akt, Wnt and other signaling pathways and IL17C, IL22 and TNF and other inflammatory factors.

Colon tissue of patients with inflammation-related colon cancer is often accompanied by high expression of inflammatory factors such as IL-6, TGF-β and TNF-α. These factors promote the occurrence and development of tumors by participating in signaling pathways related to cell proliferation and division. Studies have shown that in human colon cancer cells Caco-2 and HCT-166 systems, IL-6 and TNF-α can regulate the activation of Wnt/β-catenin signaling pathway through the NF-κB pathway; and in mice with ulcerative colitis. In cancer models, it was further found that IL-6 and TNF-α cytokines promoted tumor burden, and inhibition of TNF-α and NF-κB could partially reverse this phenomenon[15]. In this study, it was found that the expression of TNF gene decreased after HT29 cells were treated with HA. The enrichment of KEGG pathway showed that 9 genes were significantly enriched in the Wnt signaling pathway. It is speculated that HA may regulate the Wnt signaling pathway by inhibiting the expression of TNF in HT-29 cells. activation, reducing the risk of cancer. This study found that there was no significant difference in the expression of IL-6 between the blank group and the experimental group, which may be caused by the difference in cell types.

Inflammatory factors can lead to the survival and proliferation of tumor cells through local inflammation, form an immunosuppressive microenvironment that is conducive to tumor cell growth, and promote tumor angiogenesis through a series of angiogenic factors, promoting tumor growth and metastasis[16]. Chi Honggang et alfound that down-regulating the expression of inflammatory factors such as TNF-α, IL-6, and IL-1β can significantly reduce the number of tumors in colon cancer model mice [17]. Li Xinxin et al found that the expression level of IL-17 mRNA in colon

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cancer tissue is higher than that in adjacent normal tissue, and it is expected to use IL-17 as one of the important indicators and therapeutic targets for monitoring and early warning of colon cancer invasion, metastasis and poor prognosis[18]. Consistent with the results of this study, the mRNA expression level of IL-17C in HT29 cells treated with HA was significantly decreased ($P< 0.05$), suggesting that IL-17C may play a role in promoting the carcinogenesis of human colon cancer. The data obtained by high-throughput sequencing in this study laid the foundation for the further study of hyaluronic acid in HT-29 cells.

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