Research and Clinical Significance of the Differentially Expressed Genes TP63 and LMO4 in Human Immunodeficiency Virus-related Penile Squamous Cell Carcinoma

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Research article

Keywords: Human immunodeficiency virus-infected individuals, penile squamous cell carcinoma, TP63, LMO4, gene therapy

Posted Date: March 3rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-257990/v1

Abstract

Background: To study the differential gene expression and clinical significance in HIVIIs (human immunodeficiency virus-infected individuals) with penile squamous cell carcinoma.

Methods: At our hospital from 2019 to 2020, we selected 6 samples of HIV-related penile squamous cell carcinoma for the experimental group and 6 samples of non-HIV-related penile squamous cell carcinoma for the control group. Transcriptome sequencing of sample mRNAs was performed by high-throughput sequencing. Differential gene expression analysis, differential GO (Gene Ontology) enrichment analysis and differential KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis were carried out, and the RPKM (reads per kilobase per million reads) value was used as a measure of gene expression.

Results: A total of 2418 differentially expressed genes were obtained, of which 663 were upregulated and 1755 were downregulated (absolute value of logFC >1.0 and *p* value<0.05 FDR < 0.05). On the basis of the significance of the GO enrichment analysis, we found that the TP63 (tumor protein p63) gene was significantly upregulated and that the LMO4 (LIM domain only 4) gene was significantly downregulated in the experimental group compared with the control group. KEGG pathway analysis of the differentially expressed genes revealed that DNA replication was the most significant pathway associated with the upregulated genes and CAM (cell adhesion molecule) metabolism was the most significant pathway associated with the downregulated genes.

Conclusions: The gene expression profiles of HIV-related penile squamous cell carcinoma and non-HIV-related penile squamous cell carcinoma are significantly different and involve significant GO enrichment and KEGG metabolic pathways, and this is very meaningful for the study of NADCs (non-AIDS-defining cancers). Differential expression of genes may be an important target for the prevention of penile squamous cell carcinoma in HIVIIs.

Background

Penile cancer is rare but contributes significantly to morbidity and mortality. In Western countries, such as the United States and Europe, the total incidence in men is less than 1.0/100000 [1]. Approximately 95% of penile cancers have squamous cell histology. Currently, the 5-year OS (overall survival) rate for penile cancer is greater than 90% in the absence of inguinal lymph node metastasis but decreases to 29–51% when inguinal lymph nodes are involved, while the 5-year survival rate for pelvic lymph node metastasis is as low as 0–17% [2]. HIV-related penile cancer patients are special, except for the low 5-year survival rate of patients with penile cancer with lymph node metastasis, and the decreased immunity caused by HIV infection further affects their surgical opportunities and postoperative radiochemotherapies. Due to the low patient numbers, no prospective randomized studies are available.

AIDS, also known as acquired immunodeficiency syndrome, is associated with a series of clinical manifestations, such as opportunistic infection and malignant tumors. When the human body is infected with HIV, the number of CD4T lymphocytes continually decreases, seriously damaging the cellular immune function [3]. According to the tumor combinations, these cancers can be divided into ADCs (AIDS-defining cancers) and NADCs (non-AIDS-defining cancers) [4]. With the popularization of antiretroviral therapy, the incidence of immunosuppression-related ADCs has decreased, and tumors in HIVIIs tend to become NADCs [5]. HIVIIs often delay or give up treatment for their own malignancies due to concerns about privacy leaks and inadequate access to medical care. A Diagnosis and Treatment Center for AIDS was established at our hospital in 2014 to provide treatment help for HIVIIs and to provide an important source for the collection of NADC-relevant pathological specimens.

Individual immune function is closely related to cancer risk and treatment. The reduced immune monitoring and increased susceptibility to carcinogenic viruses in HIVIIs explain why people living with HIV are more likely to develop cancer than the general population [6]. Organ transplant patients on immunosuppressive regimens have a significantly higher risk of developing cancer than ordinary patients, and a similar phenomenon exists regarding the development of tumors due to immune deficiency in HIVIIs. This indicates that immune deficiency plays a very important role in HIVII-related carcinogenesis [7].

When the human body is infected with the HIV virus, the HIV gene is integrated into the cell. Like all retroviruses, HIV reverse transcribes its genome and permanently inserts itself into the selected chromosomal location in the infected cell. This leads to viral persistence and the long-term presence of virus-infected cells [8]. Targeted pattern cloning amplification occurs under certain

conditions in gene pathways important for virus replication and persistence. The unique integration with cancer genes does not increase over time, suggesting that HIV preferentially integrates into cancer-related genes and other genes that promote cell proliferation. It is believed that the identifications and influences of these genes play an important role in HIV. The possible molecular mechanism by which HIV infection leads to penile squamous cell carcinoma is unclear, and we believe that high-throughput sequencing technology will substantially benefit to these patients. In this study, we evaluated the genomic profile to understand oncogenes that may be associated with HIV.

Methods

From 2019 to 2020, 12 penile cancer specimens were collected from the urology department of our hospital. The pathological type was squamous cell carcinoma, and the patients were 48–75 years old. All patients were subjected to the following procedures before the operation: medical history and physical examination, chest radiography, electrocardiogram, abdominal color ultrasound, routine blood tests, blood biochemistry assessment, coagulation and T cell count detection. All cases were of the T2-3N1-2M0 pathological stage, and all patients underwent penile-sparing surgery and cisplatin + 5-fluorouracil chemotherapy. Six samples of HIV-related penile squamous cell carcinoma were selected for the experimental group, and 6 samples of non-HIV-related penile squamous cell carcinoma were selected for the control group. All pathological specimens were kept in a liquid nitrogen tank and then stored in a -80° refrigerator. Characteristics and clinical features of HIV infected/non-infected patients are presented in Table 1.

RNA-Seq library construction mainly includes three steps: total RNA extraction, RNA library construction and computer sequencing.

Total RNA extraction

The TRIzol extraction method was used to obtain high-quality total RNA. By default, the TRIzol volume is defined as "1 volume":

1. After adding TRIzol, the cell or tissue samples were placed at room temperature for 5 min and centrifuged at 12,000 RPM for 5 min, and the precipitate was discarded.
2. Next, a 0.2 volume of chloroform was added, and the mixture was oscillated and placed at room temperature for 15 min. The solution was then centrifuged at 12,000 g at 4℃ for 15 min, and the upper aqueous phase was removed and placed into another centrifuge tube.
3. Isopropyl alcohol was added at a 0.5 volume, and the solution was mixed well and placed at room temperature for 5-10 min. The mixture was centrifuged at 12,000 g at 4℃ for 10 min, after which the supernatant was removed, and the RNA sank to the bottom of the tube.
4. Next, 1 volume of 75% ethanol was added, and the mixture was gently oscillated in a centrifuge tube. The precipitant was suspended and centrifuged at 4℃ for 5 min at 8,000 g, and the supernatant was discarded to the greatest extent possible.
5. An appropriate amount of TE buffer was added to dissolve the RNA samples after drying at room temperature or vacuum drying for 5-10 min.
6. A NanoDrop was used to quantify the RNA concentration, and 1% agarose was used for gel electrophoresis to observe whether the bands were intact.

RNA library construction

The matching sequencing library must be constructed by using an Illumina sequencer for high-throughput sequencing. The main construction processes of the mRNA-seq library are as follows (Fig 1):

RNA-Seq analysis process

Large amounts of sequencing data are generated by high-throughput sequencing, and important information is obtained from the data by means of biological information analysis. The main bioinformatics analysis protocols for transcriptome sequencing are as follows (Fig 2):

Quality control of RNA andraw data

The total RNA concentration was determined by NanoDrop OneC and the degradation of RNA samples was detected by agarose gel electrophoresis. The amount of RNA in all samples was more than 1 ug, and the OD260/280 value of the samples was more than 1.8, which indicated that the samples had no pollution and met the requirements of database construction. The electrophoresis results showed that the rRNA bands of the samples were clear without dragging, indicating that the RNA integrity was good and met the requirements of database construction.

The original sequencing sequence of each sample was obtained after the sequencing data was downloaded, which we called raw data, stored in FASTQ file format. We used software FastQC to evaluate the quality of raw data, and used software Trimmomatic to clean the raw data.

Identification of differentially expressed genes

We compared the experimental group with control group by using the DESeq 2 R package (Version 1.27.19; http://www.bioco nduct or.org/packa ges/devel /bioc/html/DESeq 2.html) to identify the the differentially expressed genes, its strict threshold as absolute value of logFC > 1.0 and and $p < 0.05$ FDR < 0.05. Then we draw the heat map and volcano map using the heatmap R package and ggpubr R package in R software (Version 3.6.0; https://www.r-project.org/).

Analysis of GO enrichment and KEGG pathway for differentially expressed genes

After raw data were obtained, clean data were first filtered to obtain high-quality sequencing data. Clean data were compared to the reference genome of the project species to obtain comprehensive transcriptome information, and gene expression quantification, GO enrichment analysis and KEGG pathway analysis were performed.

Analysis of differential gene expression was mainly performed through GO enrichment and KEGG pathway analyses. GO is the international standard classification system of gene function. After screening the differential genes according to the purpose of the experiment, the distribution of GO terms associated with the differentially expressed genes helped to clarify the differences in gene function among the experimental samples. In organisms, different genes perform their specific biological functions through orderly coordination, and the most important biochemical metabolic and signal transduction pathways associated with differentially expressed genes can be determined by pathway significant enrichment. Hence, the abundant information in the KEGG pathway database helps to elucidate system-level biological functions of genes, such as metabolic pathways, transmission of genetic information and cellular processes, which greatly improves the practically and applicable value of the database.

Results

We used the absolute value of logFC > 1.0 (logFC is log2FC, FC is the change in multiple RPKM values of the same gene in different samples) and $p < 0.05$ FDR < 0.05 as standards to indicate that the genes were differentially expressed. We identified 663 upregulated genes and 1755 downregulated genes (Fig. 3).

The GO functional enrichment analysis showed that compared with the genomic background, the GO functional items were significantly enriched among the differentially expressed genes, and the biological functions were significantly related to the differentially expressed genes. The GO enrichment analysis of the differentially expressed genes is presented in Fig. 4. The KEGG database helps us to understand the biological functions of genes at the system level, and the KEGG pathway analysis of differentially expressed genes is presented in Fig. 5.

On the basis of the significance of GO enrichment, the most significantly upregulated term in the experimental group compared with the control group was epidermal development, and the GO code was 0008544. The GO code 0008544 consisted of a group of genes in a pathway, but among the 2418 differentially expressed genes identified in our experiment, only TP63 gene was included. The most significant downregulated term was regulation of cell activation, and the GO code was 0050865. Only the LMO4 gene matched both the genes contained in this GO code and the 2,418 differentially expressed genes we obtained. (Table 2)

KEGG pathway analysis of the differentially expressed genes between the experimental and control groups revealed that DNA replication was the most significant pathway associated with the upregulated genes and that CAM (cell adhesion molecule) metabolism was the most significant pathway associated with the downregulated genes.

Discussion

HIV accessory genes are known to extensively alter the internal composition of infected cells by hijacking normal phosphorylation and ubiquitin processes, mediating viral gene transcription, and suppressing immune surveillance and detection [8]. These data suggest that the presence of genes associated with these pathways may be associated with HIV-infected cells.

The TP63 gene belongs to a P53 family of transcription factors that also includes P73 and the tumor suppressor gene P53 [9], which share a high degree of homology and are important for cell homeostasis [10]. TP63 is an important marker for the development of basic tumor biology by regulating genetic processes [11, 12, 13]. TP63 can be synthesized from two different start sites, leading to two different N-terminal protein domains, the TA (transactivation) domain and DN domain. Both the TAp63 and DNp63 isoforms have α, β and γ splice variants in the carboxyl terminal region, which leads to multiple TP63 isoforms [14]. These isoforms play a corresponding role in regulating tumor development and other physiological and pathological processes [15]. DNp63 can act as a sequence-specific transcription activator or suppressor. DNp63α isomers, in detail, can activate specific target genes via a second transcription activation domain [16]. In addition, DNp63 interacts with a variety of epigenetic factors to effectively repress transcription [17]. TP63 is considered a transcriptional regulator of the basal gene program and is upregulated in basal subtypes of bladder, breast and ovarian cancers [12]. The enrichment of immune pathways in TAp63-expressing tumors suggests a link between TAp63 and tumor immune infiltrates [18]. An aggressive basal subtype of bladder cancer can be identified by the activation of TP63-driven genetic programs, which share the same molecular characteristics as squamous tumors occurring in other organs [19]. The superenhancer-driven long noncoding RNA LINC01503, regulated by TP63, is overexpressed and oncogenic in squamous cell carcinoma [20].

LMO4 belongs to the LIM-only family of transcriptional coregulatory proteins and consists of two LIM protein-protein interaction domains that act as connexins in multiprotein complexes [21]. Sequence analysis of the mouse LMO4 gene revealed that it spans approximately 18 kb and consists of at least six exons, including two alternatively spliced 5' exons. SLK (Ste20-like kinase) is a kinase that plays a key role in cell migration and focal adhesion turnover [22]. Mechanistically, SLK functions through complex phosphorylation regulation [23]. LMO4 can directly bind to SLK in vivo and in vitro and activate its kinase activity [24]. LMO4 can also interact with Ldb1 (LIM domain-binding protein) and play a role in cell proliferation and motility [25]; for example, LMO4 is overexpressed in samples of patient tissue and oral squamous cell carcinomas [26]. Deletion of LMO4 impairs Ldb1 and SLK recruitment in migratory cells [24]. LMO4 is closely related to the occurrence of many malignant tumors, such as pancreatic cancer, non-small cell lung cancer, and head and neck cancer [27, 28], and can affect the differentiation of T cells, leading to acute leukemia [29].

The above results demonstrate that the TP63 and LMO4 genes may change the internal composition of HIV-infected cells by mediating viral gene transcription and phosphorylation pathways. Moreover, HIV-encoded proteins may interact with the TP63 and LMO4 proteins and modulate their function in different ways. TP63 and LMO4 have been intensively studied as transcriptional targets in squamous cell carcinoma. They can regulate the expression of diverse tumor-related proteins and are involved in extracellular matrix and tumor microenvironment remodeling as well as in growth factor-mediated signal transduction [30, 31, 32]. Pathological specimens of HIV-related penile squamous cell carcinoma are very rare, so our sample size is relatively small, but the results obtained still have certain reference value. First of all, the heterogeneity between the included cases was very low, including the pathological types and stages of penile squamous cell carcinoma in the experimental group and the control group, as well as the surgical and chemoradiotherapy methods adopted. Secondly, we carried out quality control of RNA and raw data, and found that the differentially expressed genes were significant (absolute value of logFc > 1.0 and p value < 0.05 FDR < 0.05). Our small sample size made it difficult to compare the genomic locations of TP63 and LMO4 mutations, and whether these differences in presentation and prognosis are related to the systemic effects of HIV-mediated immunosuppression or to specific biological characteristics of the primary tumor is still unclear. Additional studies with larger sample sizes are needed to validate our experimental results. Genetic studies on NADCs provide another possibility and opportunity to explore the relationship between HIV and tumors in regards to biological processes and molecular pathways. Whether the inhibition of related pathways or genes can induce immune reconstruction to combat early penile cancer and prevent the occurrence of NADCs is worthy of further study.

Among the KEGG pathways, two terms linked to the regulation of cell proliferation and survival, including DNA replication and cell adhesion molecule metabolism, were identified. This suggests that HIV integration may promote the proliferation and persistence

of infected cells. Although HIV has not been shown to directly cause carcinogenic transformation, the incidence of cancer in HIVIIs is higher than that in patients without HIV. HIV can persist and permanently insert itself into selected chromosomal locations within infected cells; it can also reverse transcribe its genome, like all retroviruses. Once HIV successfully integrates into a chromosomal site, all subsequent cells generated by cell division contain the same viral integration site [33]. Approximately 80% of the integration sites have viral integration within gene transcripts, and approximately 12.5% of these genes are related to cancer development. It is unclear whether a link exists between the HIV site selection and potential oncogenic development, but Wagner TA et al. found that HIV preferentially integrates into cancer-related genes and other genes that promote cell proliferation [34]. There is also the idea that HIV DNA is undetectable in the cancer cells of most HIVIIs; however, experiments supporting this idea are not well documented in the literature, and many HIV proviruses may have been missed by testing only a small fraction of the HIV genome [35].

After HIV infection, the virus can have a synergistic effect on tumorigenesis, and HIV-1 (human immunodeficiency virus type 1) is among the most common synergistic oncogenes [36]. HIV-1 is a terrible pathogen that can cause persistent infections and evolve rapidly by integrating the original viral genome into chronically infected cells [37]. During viral replication, HIV-1 can mutate and recombine at a high frequency, promoting viral persistence and regulating HIV replication while usurping the cellular mechanisms of HIV replication during gene expression [38]. Further research on HIV-1 replication may help to identify new targets for antiretroviral therapy that will enable continued viral inhibition in patients with treatment failure [39]. The TAT (transactivator of transcription) proteins produced by the HIV-1 gene, regardless of their immune status, are widely associated with the increases in angiogenesis and other functions in ADCs and have become the research target for a treatment strategy. G Saraga et al prepared HIV-1 moderators, which have potential as mediators and have been used in clinical gene therapies for pancreatic cancer [40]. HIV-based lentiviral vectors and many other gene delivery strategies have been used to evaluate cell culture, small and large animal models, and HIV treatment methods in patients [41].

Genome-wide techniques have been used in the field of HIV research to achieve the goal of understanding the complex interactions between host and pathogen [42]. The expression and transcription of HIV-1 gene is a key step in the viral replication cycle and is considered as a potential target for inhibition of HIV-1 [43]. Liang Shan et al. found that in the analysis of the regulation of HIV-1 gene expression, it was important to consider the nature of HIV-1 integration sites. Reactivation of latent HIV-1 might be a strategy to eliminate the HIV-1 latent reservoir in resting memory CD4(+) T cells. By compared viral integration sites and the levels of expression of the host gene, they had identified new features of the integration sites of latent HIV-1, which might provide a better understanding of the mechanism of HIV-1 latency [44].

In recent years, the use of transgenic T cells to treat previously incurable diseases such as cancer has multiplied. This success is now driving the use of the same technology to treat HIV infection [45, 46]. While antiretroviral combination therapy can significantly reduce the circulating viral loads in HIV-infected patients, highly replicable viruses remain. Thor A Wagner et al noted that mechanisms allowing the persistence of HIV include long-term latent infection of cells, low levels of HIV replication, and proliferation of HIV-infected cells. Integrating HIV into specific genes may promote the proliferation of HIV-infected cells and slow the decay of the virus in antiretroviral therapeutic processes [47]. Of all the methods currently being studied to treat HIV, the effectiveness of gene therapy is supported by most data. Gene therapy has the advantages of specificity and persistence and has the potential to protect patients from subsequent infections. A patient in Berlin that was positive for both HIV and AML (acute myeloid leukemia) received two stem cell transplants from a donor homozygous for a CCR5delta32 mutation. Eight years after his second transplant, he still had no HIV or AML infection. This case provides a strong proof-of-principle that a cure for HIV is possible and can be achieved through gene therapy [48]. Some articles have pointed out that in genome-wide studies, HIV integration is beneficial for the transcription sites of active genes and for the establishment of HIV replication and latency [49, 35], thus promoting pathways related to tumorigenesis [34]. Understanding the relationship between HIV and tumors may provide a method for reducing the risk of HIV tumors or even a genetic pathway for treating HIV. As the principle suggests, gene therapy can cure HIV, and the improvement of current methods may lead to the optimization of transduction efficiency and persistence in vivo. Gene therapy strategies are linked to human pathology at a fundamental level to correct and improve the underlying genetic factors of any disease by delivering DNA and RNA molecules. The history of HIV gene therapy is particularly interesting, as targeted viruses are quickly chosen together as part of targeted strategies. It is generally accepted that the combination approach is the most promising for the functional treatment of HIV infection.

Conclusion

Gene therapy for HIV has extremely great potential. Herein, we evaluated genomic profiles to better understanding oncogenic genes that may be associated with HIV. These results may provide a genetic pathway to reduce the risk of NADCs and even provide more ideas for HIV treatment. The differentially expressed genes TP63 and LMO4 may be targets to prevent the development of penile squamous cell carcinoma in HIVIIs.

Abbreviations

HIVIIs: Human immunodeficiency virus-infected individuals; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; RPKM: Reads per kilobase per million reads; TP63: Tumor protein p63; LMO4: LIM domain only 4; CAM: Cell adhesion molecule; NADCs: Non-AIDS-defining cancers; ADCs: AIDS-defining cancers; OS: Overall survival; TA: Transactivation; SLK: Ste20-like kinase; Ldb1: LIM domain-binding protein; HIV-1: Human immunodeficiency virus type 1; TAT : Transactivator of transcription; AML: Acute myeloid leukemia.

Declarations

Ethics approval and consent to participate

The research was reviewed and approved by the Ethics Committee of Beijing Youan Hospital Capital Medical University. The Ethics Committee archive number is LL-2019-176-K, and the approval number is [2020]035.

All the participants provided informed consent.

Consent to publish

Written informed consent for publication was obtained.

Availability of data and materials

The datasets generated during and/or analysed during the current study are not publicly available due patients privacy but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by China Primary Healthy Care Foudation - Youan Foudation of Liver Disease and AIDS. Registration number: YNKTQN20180212. The funders had no role in the study design and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

WRX was a main contributor in the design, implementation and writing of the manuscript. XPH and XZ did the experiments and data collection. YZ contributed much in the revised version of our manuscript for updating the literature and revising the paper. All authors read and approved the final manuscript.

Acknowledgements

The authors acknowledge Zhen Huang for his assistance in data collection and transcription.

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Tables

Table 1

Patients characteristics

Group	Patients	Age, years	Pathological type and molecular pathology	TNM	Treatment received	Follow-Up	AIDS time and $CD4^+$
Experimental Group	1	48	squamous carcinoma and HPV high risk	T2N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	9 months FU, No recurrence or metastasis	5 years, 386/ul
	2	64	squamous carcinoma and HPV high risk	T3N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	12 months FU, No recurrence or metastasis	5 years, 449/ul
	3	57	squamous carcinoma and HPV high risk	T3N2M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	14 months FU, No recurrence or metastasis	1 years, 421/ul
	4	62	squamous carcinoma and HPV high risk	T3N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	12 months FU, No recurrence or metastasis	1.5 years, 359/ul
	5	69	squamous carcinoma and HPV high risk	T3N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	13 months FU, No recurrence or metastasis	3.5 years, 383/ul
	6	64	squamous carcinoma and HPV high risk	T3N2M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	10 months FU, No recurrence or metastasis	2 years, 490/ul
Control Group	1	75	squamous carcinoma and HPV high risk	T2N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	15 months FU, No recurrence or metastasis	NA, 568/ul
	2	59	squamous carcinoma and HPV high risk	T3N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	7 months FU, No recurrence or metastasis	NA, 900/ul
	3	70	squamous carcinoma and HPV high risk	T3N2M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	12 months FU, No recurrence or metastasis	NA, 747/ul
	4	66	squamous carcinoma and HPV high risk	T2N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	13 months FU, No recurrence or metastasis	NA, 691/ul
	5	49	squamous carcinoma and HPV high risk	T3N2M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	14 months FU, No recurrence or metastasis	NA, 554/ul
	6	51	squamous carcinoma and HPV high risk	T3N1M0	glans amputation and chemotherapy with cisplatin + 5-fluorouracil	7 months FU, No recurrence or metastasis	NA, 611/ul

Table 2

GO and KEGG pathway enrichment analysis of the differentially expressed genes

Terms		Database	ID	Input number	Backgroundnumber	Rich factor	P Value	Corrected P Value
Upregulated	epidermis development	GO biological process	GO:0008544	47	298	4.85	1.71E-16	1.02E-12
	DNA replication	KEGG PATHWAY	hsa03030	14	36	11.96	4.93E-10	1.18E-07
Downregulated	regulation of cell activation	GO biological process	GO:0050865	142	463	3.56	6.73E-24	5.23E-20
	Cell adhesion molecule (CAM)	KEGG PATHWAY	hsa04514	57	146	4.54	1.66E-11	4.36E-09

Figures

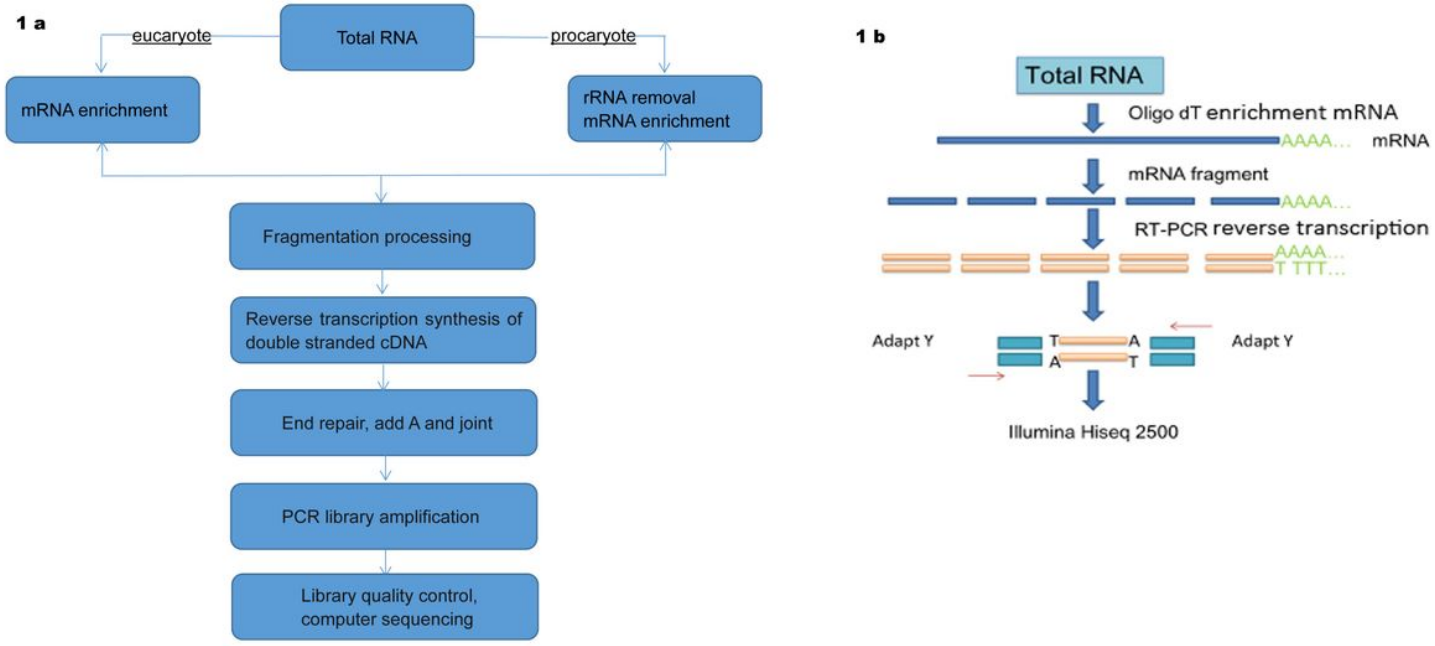

Figure 1

mRNA-seq library construction process

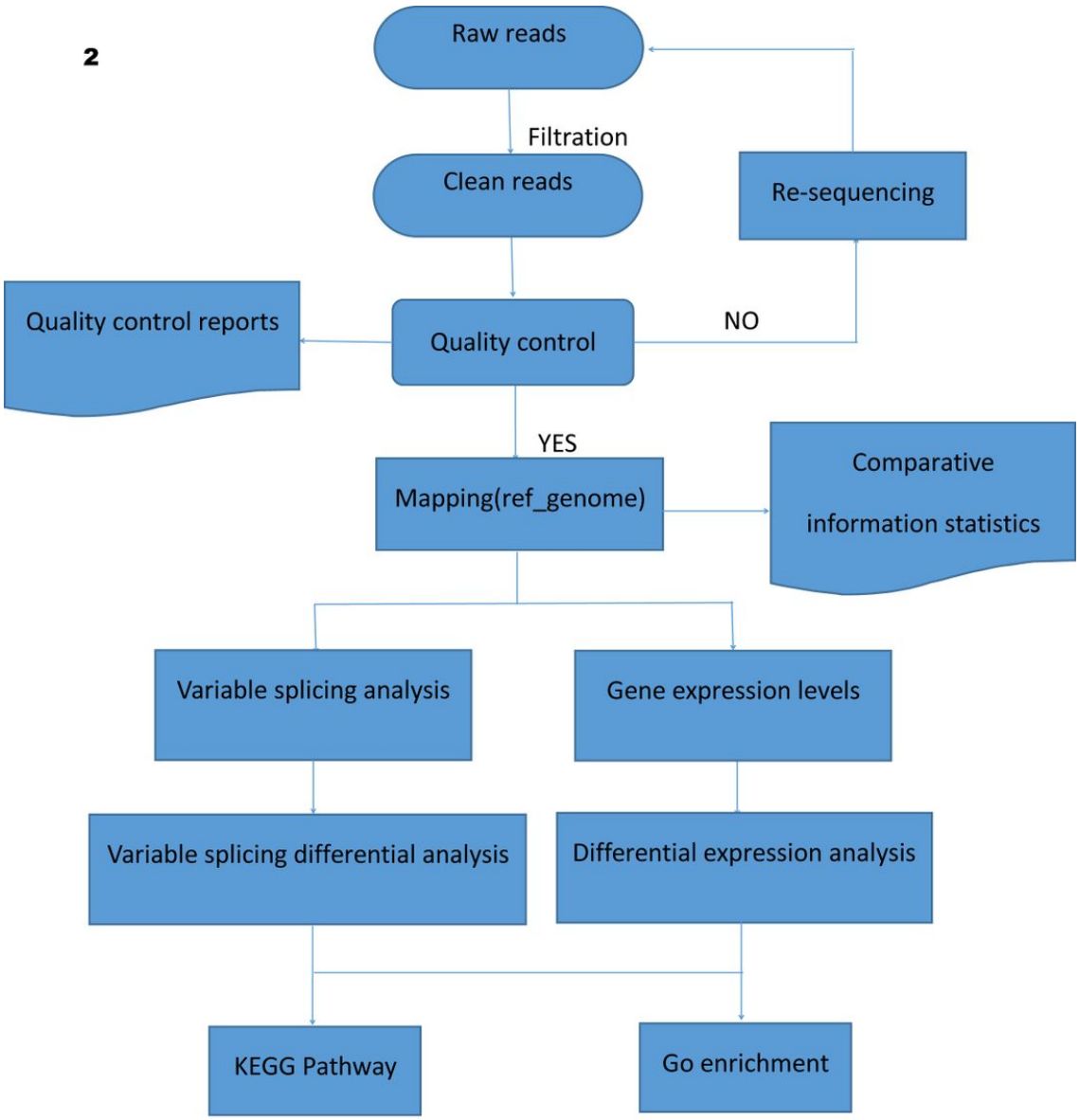

Figure 2

Analysis process of RNA-Seq

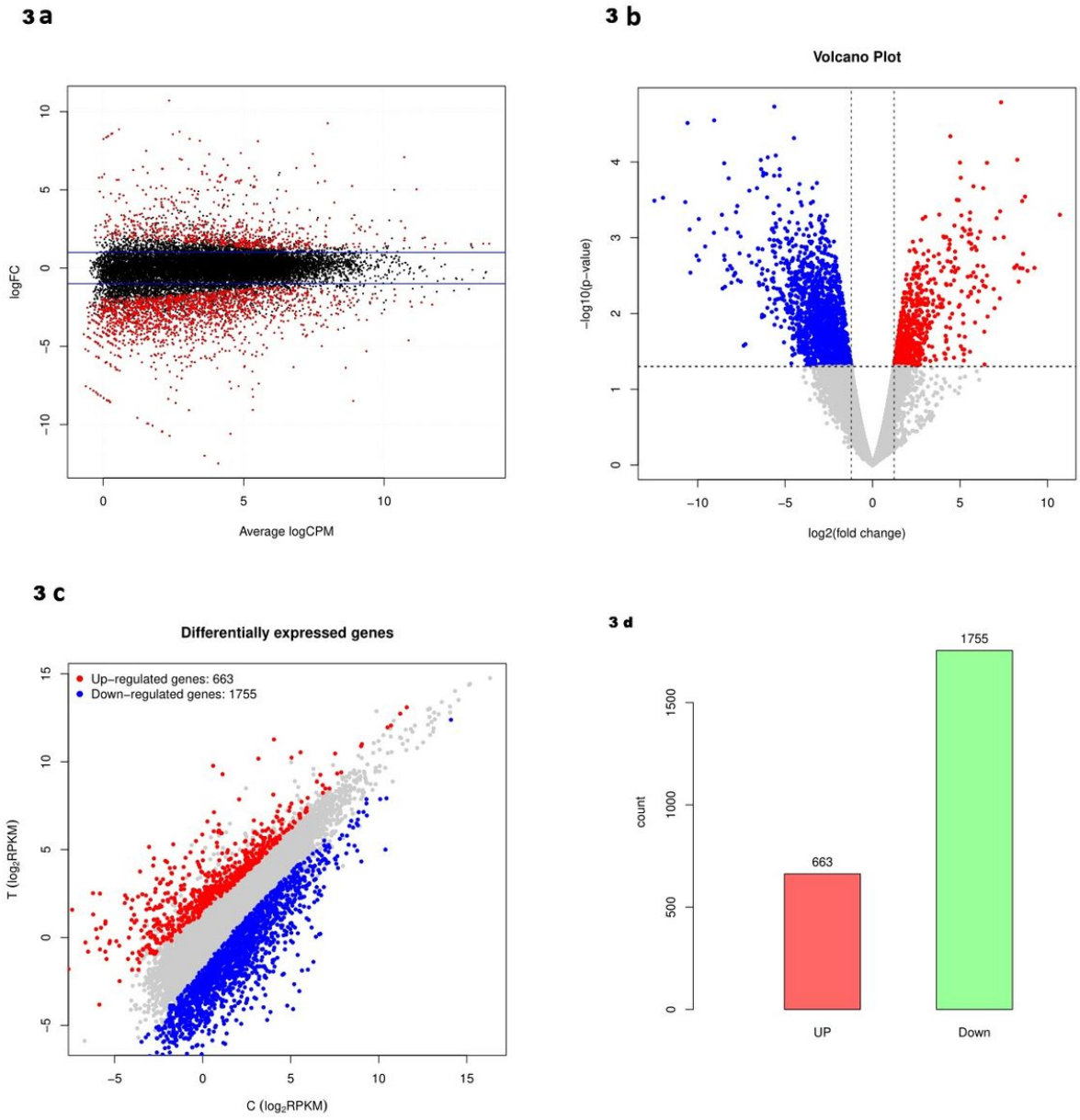

Figure 3

3a. MA Plot of the differentially expressed genes. Average log CPM: Average log2 counts-per-million; logFC: log-fold-changes between each pair of RNA samples; Two blue lines representing the up-down threshold for differentially expressed genes (logFC>1 indicates upregulated, logFC< -1 indicates downregulated). 3b. Volcanic map of the differentially expressed genes. Log2(fold change): The logarithm of the difference multiples based on 2; -log10(p-value): p value negative logarithm based on 10; The gray dots represent genes that are not differentially expressed, the blue dots represent genes that are differentially downregulated, and the red dots represent genes that are differentially upregulated. 3c. Scatter plot of expression between comparison groups. The horizontal and vertical coordinates represent the experimental group and the control group respectively, a logarithm of the expression RPKM based on 2. The gray dots represent genes that are not differentially expressed, the blue dots represent genes that are differentially downregulated, and the red dots represent genes that are differentially upregulated. The upper left corner indicates the number of upregulated and downregulated genes. 3d. Statistics on upregulated and downregulated of the differential expression genes

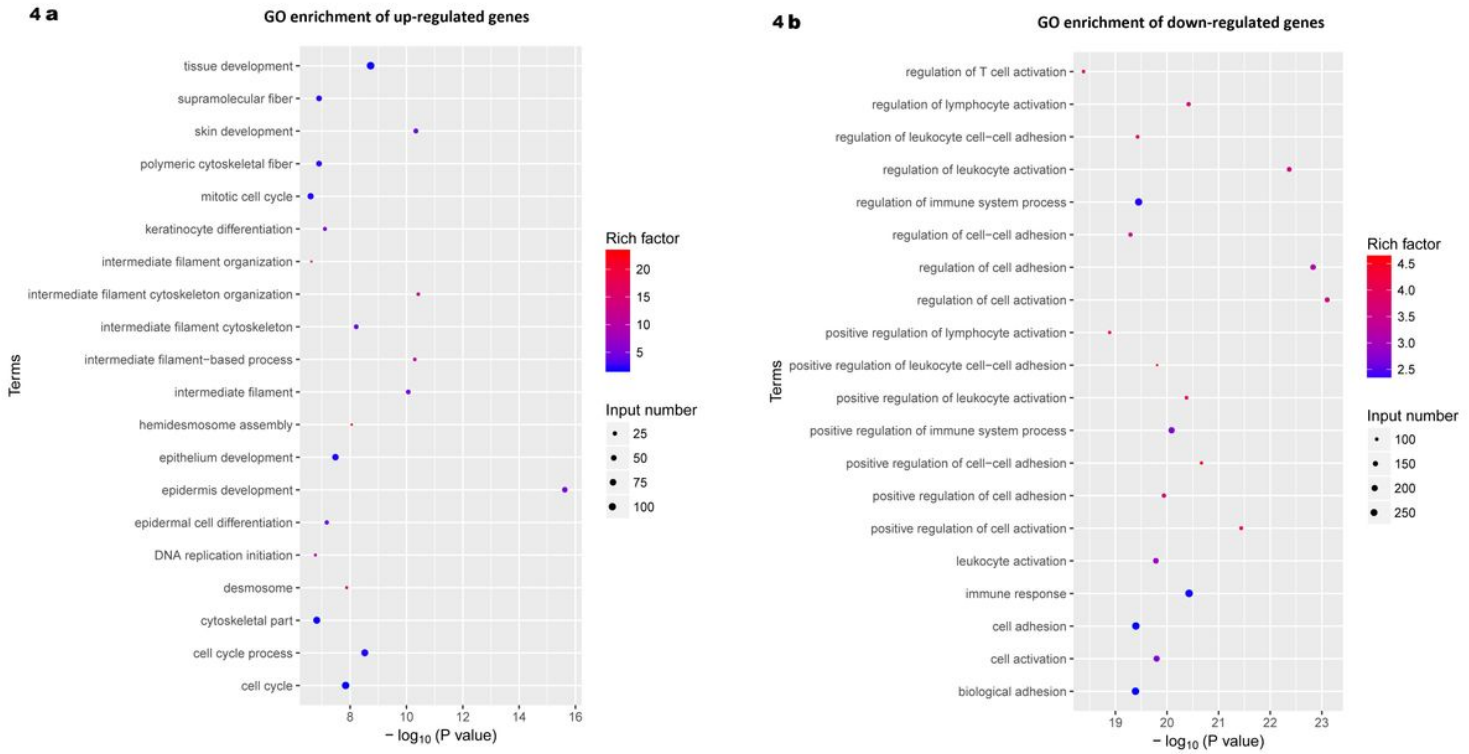

Figure 4

The horizontal axis indicates the significance of enrichment, expressed as -log10, the large the value, the more significant the enrichment. The longitudinal axis represents the GO Terms of enrichment. The dot size indicates the number of different genes contained in the GO Terms, and the dot depth indicates the degree of rich factor enrichment.

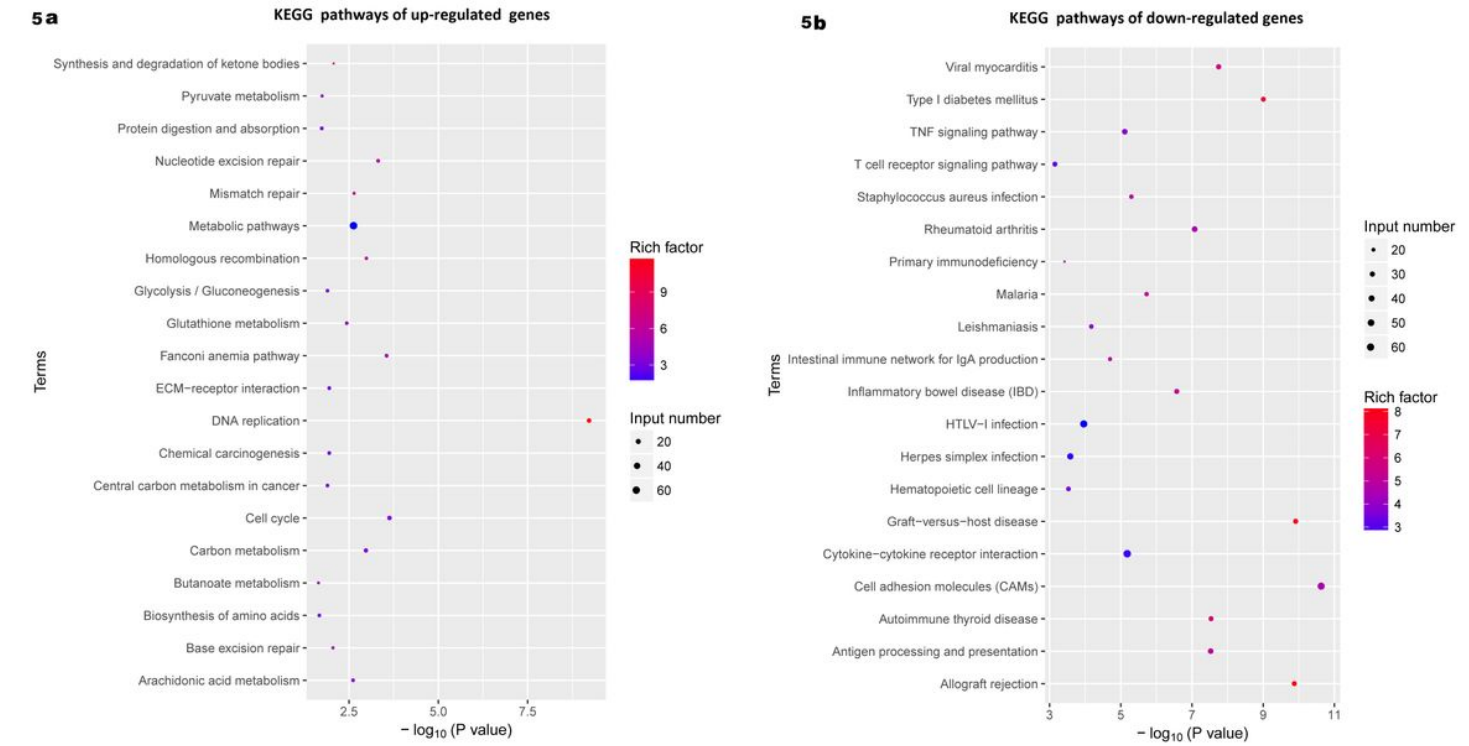

Figure 5

The horizontal axis indicates the significance of enrichment, expressed as -log10, the large the value, the more significant the enrichment. The longitudinal axis represents the KEGG pathways. The dot size indicates the number of different genes contained in the KEGG pathways, and the dot depth indicates the degree of rich factor enrichment.