

RNA-sequencing Reveal the Molecular Mechanism of Moxibustion on Neutrophil Gene Expression Profile During Breast Cancer Chemotherapy

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Research

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Abstract

Background

Moxibustion is a Traditional Chinese Medicine (TCM) therapy that can prevent neutropenia after breast cancer (BC) chemotherapy. We aimed to explore the mechanism of moxibustion for treating BC chemotherapy-induced neutropenia (CIN).

Methods

Fifty patients with BC undergoing chemotherapy and who met the inclusion criteria were randomized into an intervention (IT) group and control (OC) group. After excluding cases, there were 14 cases in the RNA sequencing (RNA-seq) group and 17 cases in the verification group. Neutrophils were extracted from the peripheral blood of the patients in both groups before and after chemotherapy. RNA-seq and bioinformatics analysis were performed in RNA-seq group. The verification group were verified using real time quantity polymerase chain reaction (RT-qPCR).

Results

RNA-seq screened 1092 DEGs before and after chemotherapy in the OC group, and 571 DEGs in the IT group. Compared with the OC group, the IT group had 707 DEGs after chemotherapy. The effect of moxibustion on the patients' neutrophil gene expression profiles was related to the cell adhesion, adaptive immune response and metabolic pathways, and leukocyte migration, etc. The co-network results showed that TSC22D1 and TGFB111 have core regulatory function. GO and KEGG enrichment pathway analysis suggested a close correlation with the TGF β 1/TSC22D1-mediated cell adhesion molecules (CAMs) pathway. RT-qPCR showed that CD177 in the neutrophils was significantly upregulated after chemotherapy compared with that before chemotherapy ($P < 0.05$) without moxibustion, while TSC22D1 showed a downward trend ($P = 0.094$). Moxibustion significantly increased the expression of TSC22D1, ANKFY1, and ITGB3 in the neutrophils ($P < 0.05$) after BC chemotherapy.

Conclusion

The mechanism of moxibustion in improving CIN may be related to the regulation of the TSC22D1 expression profile in neutrophil. We present a novel insight into the mechanism of moxibustion treatment of CIN in patients with BC.

Trial registration

Chinese Clinical Trial Register, ChiCTR-INR-16009557. Registered 23 October 2016, <http://www.chictr.org.cn/showproj.aspx?proj=16203>.

Background

Breast cancer (BC) is the most common malignant tumor in Chinese women, accounting for 12.2% and 9.6% of new cancer cases and deaths worldwide, respectively (1). BC is one of the most effective tumors in the application of chemotherapy in solid tumors, so chemotherapy plays an important role in the comprehensive treatment of BC. Patients with early-stage BC are often recommended dose-dense anthracycline combined with taxanes chemotherapy regimen (2). However, such a combined regimen has obvious chemotherapy-induced neutropenia (CIN) and febrile neutropenia (FN), which are the most important dose-limiting toxicities during chemotherapy (3, 4). Therefore, the mechanism and treatment of neutropenia have become a critical topic in BC. Moxibustion is a Traditional Chinese Medicine (TCM) therapy that involves burning the herb moxa (*Folium Artemisia argyi*, or mugwort) over selected acupoints, which is intended for enhancing qi and blood and for relieving swelling and pain (5). Moxibustion can prevent leukopenia after BC chemotherapy and reduce gastrointestinal burden effectively (6-9). Furthermore, moxibustion can play a role in promoting DNA repair, affecting the proliferation cycle, interfering with the signaling pathway of bone marrow cells, and improving the microenvironment of bone marrow hematopoiesis (10, 11).

Clinical studies have found that grade 3–4 neutropenia or FN are often severe adverse events (SAE) of BC chemotherapy (12, 13), so the use of peripheral blood neutrophils as experimental test samples has the advantage of convenient materials and accurate targets. However, there are few studies on the effect of moxibustion mechanism on the neutrophil profile during BC chemotherapy.

In the present study, we used RNA-seq to analyze the differential expression of neutrophil genes, gene function, and pathway enrichment before and after moxibustion intervention. Our research provides new ideas for further exploration of the molecular mechanism of moxibustion for improving CIN in patients with BC.

Methods

Patients

Fifty patients were recruited from within our institution. The inclusion criteria were female sex, age 18–80 years, pathological diagnosis of primary invasive BC, expected to undergo chemotherapy according to the guidelines and signed informed consent for inclusion in the study. The exclusion criteria were liver and kidney dysfunction, severe heart disease, pregnancy, and the presence of other primary malignant tumors.

Treatment

Patients in the intervention (IT) group underwent moxibustion at the Shenque (CV 8), Qihai (CV 6), Guanyuan (CV 4), Zusanli (ST 36), and Sanyinjiao (SP 6) acupoints. Mild moxibustion was applied for 15 minutes per acupoint once daily. Moxibustion started on day 2 after chemotherapy, and continued every day until the day before the next chemotherapy. The control (OC) group was treated routinely with chemotherapy without moxibustion. The patients enrolled from January 2018 to December 2018 were defined as the RNA sequencing group, with transcriptome sequencing (RNA-seq). The patients enrolled

from January 2019 to September 2019 were defined as the verification group, with real-time quantitative polymerase chain reaction (RT-qPCR). The patients were all from the study (14), which has been registered at chictr.org.cn (ChiCTR-INR-16009557). The study was approved by the Yueyang Hospital ethics committee (Certificate No. 2016-070, 2016-0170-02, and 2016-070-03). We obtained written informed consent from all patients before enrollment.

Sample collection

Peripheral blood samples were collected in EDTA tubes twice (6 mL per collection) from each patient. The first collection was 1–3 days before the first course of chemotherapy (d-1~3), and was the baseline before chemotherapy (BF); the second collection was on day 20 after the first course of chemotherapy (d20), and was the blood sample after chemotherapy (AF) (Fig.1). The blood samples were drawn from the overnight-fasted patients at 9 AM: 2 mL was used for routine blood testing, and 4 mL was used for neutrophil isolation and extraction according to the manufacturer's instructions (LZS11131, TBD science, China).

Neutrophil identification

Isolated neutrophils were observed by Diff-quick staining (D030-1-1, Nanjing Jiancheng, China) according to the manufacturer's instructions. Neutrophil density was adjusted to 1×10^6 cells/mL. The cells were incubated at room temperature with PBS and anti-CD44-FITC (Abcam; cat. ab27285) and anti-CD11b-FITC (Abcam; cat. ab24874) separately, and analyzed using a flow cytometer (BD Biosciences).

RNA-seq analysis

Total RNA was isolated using TRIzol (Invitrogen, Waltham, MA, USA). mRNA libraries were generated using an RNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. All libraries were purified and pooled at 1:1 at 2 nM, and sequenced using an Illumina HiSeq platform using 150-bp paired-end reads. Data preprocessing, principle component analysis (PCA), and hierarchical clustering analysis (HCA) were performed in R statistical software (www.r-project.org) using the base function and the stat package. Differentially expressed genes (DEGs) were determined based on fold change (FC) and t-tests, where $FC > 1.5$ or < 0.67 , and $P < 0.05$. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology biological process (GOBP) analyses were used to identify the biological functions and diseases associated with DEGs via Metascape (metascape.org). We constructed gene network adjacency between genes according to Pearson's correlation. We selected the genes with high correlations (≥ 0.8) to construct a network graph using Cytoscape 3.6.1.

RNA extraction and RT-qPCR

Total RNA was isolated using TRIzol (Invitrogen). The primers were designed using Primer Premier 6.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by Generay Biotech (Table 1). RT-qPCR was performed using the KAPA SYBR Green Supermix PCR kit and AriaMx Real-Time PCR

System (Agilent Technologies, Santa Clara, CA, USA). Differences in gene expression were calculated using the comparative CT method ($2^{-\Delta\Delta CT}$) (15), and RT-qPCR was normalized according to GAPDH.

Statistical analysis

All values are expressed as the mean \pm standard deviation. Significant differences between groups were statistically analyzed using the independent t-test or nonparametric test. Significant differences before and after moxibustion in the same group were analyzed by the paired-samples t-test or nonparametric test. Discrete data were compared using the χ^2 test or an accurate probability method. IBM SPSS 20.0 software was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics and baseline measurements

Fifty patients with BC from January 2018 to September 2019 met the study criteria. We collected blood samples from all of the patients at BF; two samples were hemolyzed, and two samples were degraded. At AF, blood samples were not collected from 15 cases. There were an eventual 14 cases in the RNA-seq group: five in the IT group and nine in the OC group. There were 17 cases in the verification group: ten in the IT group and seven in the OC group. There were no significant differences between the OC and IT groups in terms of age, menstrual status, chemotherapy regimen, tumor stage, molecular typing, and blood routine test in the sequencing group and verification group (Table 2, 3).

Neutrophil identification

Diff-quick staining showed clear neutrophil granules and purple-red nuclei. CD44 and CD11b were the molecular markers of neutrophils. Anti-CD44-FITC was detected on the surface of 98.7% isolated neutrophils. Anti-CD11b-FITC was detected on the surface of 96.6% isolated neutrophils, which proved the high purity of neutrophils extracted from human peripheral blood (Fig 2).

DEG and functional analysis in OC and IT groups

PCA and HCA showed that each group had significant specificity (Fig. 3a, 3b). The DEG analysis showed that, after chemotherapy, there were 1092 DEGs in total between the OC_AF and OC_BF groups, 571 DEGs between the IT_AF and IT_BF groups, and 707 DEGs between the OC and IT groups (Fig. 3c).

In the OC_AF group as compared to the OC_BF group, the upregulated genes included CD177, PTX3, and AZU1, and the downregulated genes included *ITGB1*, *TSC22D1*, and *ITGB3*. GOBP and KEGG pathway analysis showed that the RNA transport, translation and metabolic pathways were significantly enriched (Fig. 3d).

The effect of moxibustion on the neutrophil transcriptome was analyzed to gain insight into the potential mechanism of moxibustion in the efficacy of CIN. In the IT group, the [ANKFY1](#), [ATP11A](#), and [TJP2](#) genes were upregulated after moxibustion, whereas [TK1](#), [TRAPPC2L](#), and [SNHG8](#) were downregulated. The translation, adaptive immune response, cell adhesion and metabolic pathways were significantly enriched by GOBP and KEGG pathway analysis (Fig. 3e).

DEG analysis related to moxibustion regulation of neutrophils and network analysis

To explore the molecular mechanisms of moxibustion regulation of neutrophils after chemotherapy, RNA-seq was performed to analyze the DEG profiles, gene function, and signaling pathways between the IT and OC groups after chemotherapy (OC_AF vs. IT_AF). Moxibustion after chemotherapy upregulated the expression of [TSC22D1](#), [ITGB3](#), and [GFI1B](#), and downregulated [PRKCZ](#) and [TK1](#). The signaling pathways, including cell adhesion, leukocyte migration, and cell cycle, were significantly enriched (Fig. 4a). The co-network revealed that most DEGs were regulated by $TGF\beta 1$ and [TSC22D1](#). KEGG enrichment pathway analysis suggested a close correlation with the $TGF\beta 1$ /[TSC22D1](#)-mediated cell adhesion molecules (CAMs) pathway, in which adhesion molecules such as [ITGB1](#), [ITGB3](#), [JAM3](#), [CLDN5](#), and [ESAM](#) were significantly up-regulated (Fig. 4b).

Transcription factors (TF) such as [FOSL1](#), [ZNF274](#), [E2F4](#), [PRRX2](#), [ZNF284](#), [HHEX](#), [CEBPG](#), and [ZBTB2](#) have regulatory effects on DEGs, of which [FOSL1](#) has the most negative regulatory effect. In addition, [LILRB3](#) and [LILRB4](#) are jointly regulated by TF such as [HHEX](#), [ZNF284](#), and [PRRX2](#) (Fig. 4c). To investigate the potential functional implication of the TF network, we performed functional enrichment analysis for all genes in the network based on GO terms. Based on the P-values, the enriched GO BP terms included blood coagulation, platelet degranulation, cell–matrix adhesion, leukocyte migration, and neutrophil homeostasis. The enriched KEGG included platelet activation, cell adhesion molecules (CAMs), vascular smooth muscle contraction, and antifolate resistance (Fig. 4d).

RT-qPCR verification

RNA-seq results suggested up-regulation of [CD177](#) expression and down-regulation of [ITGB1](#) and [TSC22D1](#) in the OC_AF group compared to the OC_BF group. [ANKFY1](#) and [ATP11A](#) were up-regulated and [TK1](#) was down-regulated in the IF_AF group compared to IT_BF group. In addition, [TSC22D1](#) and [ITGB3](#) were up-regulated and [TK1](#) was down-regulated in the IT_AF group compared to OC_AF group. Therefore, we validated the gene expression of [CD177](#), [ITGB1](#), [TSC22D1](#), [ANKFY1](#), [ATP11A](#), [TK1](#) and [ITGB3](#) by RT-qPCR.

Without moxibustion, [CD177](#) gene expression in neutrophils after chemotherapy was significantly higher than that before chemotherapy (Fig. 5a), while the expression of [TSC22D1](#) showed a downward trend ($P = 0.094$), and [ITGB1](#) have no significant differences (Fig. 5b). Under the intervention of moxibustion, [ANKFY1](#), [TSC22D1](#) and [ITGB3](#) expression was significantly upregulated ($P < 0.05$) (Fig. 5c-e). [ATP11A](#) and [TK1](#) have no significant differences (Fig. 5f).

Discussion

Bone marrow suppression caused by BC chemotherapy often affects the implementation of chemotherapy (16, 17). BC chemotherapy destroys hematopoietic function, mainly manifested by decreased absolute neutrophil count (ANC) (18). Neutrophils can secrete many cytokines, and recruit and regulate other immune cells, thereby regulating the inflammatory response (19). Therefore, how to increasing neutrophils plays an important role in BC chemotherapy.

According to TCM theory, moxibustion at CV 4, CV 6, CV 8, ST 36, and SP 6 can regulate the spleen and stomach, benefit the liver and kidneys, warm the yang and strengthen qi, replenish blood, produce marrow, and reduce inflammatory cytokine expression (20-22). Multiple TCM studies have shown that moxibustion has a good regulatory effect during chemotherapy in patients with BC (23, 24).

Moxibustion can improve CIN of BC, the mechanism may be related to the regulation of neutrophil gene expression profile. In the present study, RNA-seq showed that moxibustion upregulated the expression of TSC22D1, ANKFY1, and ITGB3 in neutrophils after BC chemotherapy, including cell adhesion, adaptive immune response, metabolic pathways, and leukocyte migration. TSC22D1 plays a central role in moxibustion intervention for BC chemotherapy. The co-network revealed that most DEGs were regulated by TGF β 1 and TSC22D1. KEGG enrichment pathway analysis suggested a close correlation with the TGF β 1/TSC22D1-mediated cell adhesion molecules (CAMs) pathway, in which adhesion molecules such as ITGB1, ITGB3, JAM3, CLDN5, and ESAM were significantly up-regulated. TSC22D1 is a member of the TSC22/Dip/Bun family. It is stimulated by TGF- β 1 and regulates the transcription of various genes, including that for C-type natriuretic peptide (CNP) (25). Ohta et al (26) found that TSC22D1 protein may play a key role in tumor suppression by inducing cancer cell apoptosis. Choi et al (27) found that TSC22D1 can be combined with Smad4 to enhance the TGF- β signaling pathway, indicating that TSC22D1 can be used as a transcription regulator in combination with other TF to regulate the transcription of target genes. In breast-related diseases, TSC22D1 plays an important role in oncogene-induced cellular senescence (OIS). TSC22D1 is downregulated with cyclin p15 inhibition (28).

Conclusions

We established a BC chemotherapy moxibustion intervention cohort to study the mechanism of moxibustion for improving CIN in patients with BC. This study provides a new target for studying the mechanism of moxibustion treatment of CIN.

Abbreviations

TCM, Traditional Chinese Medicine;

BC, breast cancer;

CIN, chemotherapy-induced neutropenia;

IT, intervention;

OC, control;

RNA-seq, RNA-sequencing;

RT-qPCR, real time quantity polymerase chain reaction;

CAMs, cell adhesion molecules;

GOBP, Gene ontology biological process;

KEGG, Kyoto Encyclopedia of Genes and Genomes;

Declarations

Ethics approval and consent to participate

The study was approved by the Yueyang Hospital ethics committee (Certificate No. 2016-070, 2016-0170-02, and 2016-070-03).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests

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Authors' contributions

YJJ, XHX and HGW designed the research, analyzed the data, and wrote the paper. YJJ, XYZ, SYL, SYS, WLC, YL and YH enrolled patients, collected samples and performed experiments. All authors reviewed the results and approved the final version to be submitted.

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Tables

Table 1. The sequences of the primers.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	GCAAGTTCAACGGCACAGTCA	ACGACATACTCAGCACCAGCAT
ANKFY-1	AGCAGCGAGTCCTTCATCAG	ATCCAGCGAAGCATTGTCATC
ATP11A	ACCAGATCCGAGCCAGAGT	TGTAGCACCAAGCAGAGTAAGA
CD177	CCTGATCTCCTACACCTTCGT	TCCAGACAGCCTTCCATAGAC
ITGB1	TCAGATCCAACCACAGCAGTT	TGAAGTCCGAAGTAATCCTCCT
ITGB3	TGAGGAAGTGAAGAAGCAGAGT	GGTAGTGGAGGCAGAGTAATGA
TK1	TCGCTTCCAGATTGCTCAGTA	CAGCCACAATTACGGTCTTCC
TSC22D1	GTGGCGATGGATCTAGGAGTT	CACTTCTTCTCTGACCGCATAC

Table 2. Demographic Characteristics and Baseline Measurements of sequencing group (N=14).

Characteristic	IT (N=5)	OC (N=9)	t/ χ^2/Z	P- value
Age	26~75 (54.5±10.1)	33~70 (55.1±9.7)	-0.276	0.783
Menstrual status				
Premenopausal	1 (20.0)	2 (22.2)	0.090	0.725
Postmenopausal	4 (80.0)	7 (77.8)	0.090	0.725
Chemotherapy regimen				
Anthracyclines only	0 (0)	0 (0)	-	-
Taxanes only	1 (20.0)	2 (22.2)	0.090	0.725
Anthracycline combined with taxanes	4 (80.0)	7 (77.8)	0.090	0.725
Tumor stages				
I	1 (20.0)	4 (44.4)	0.837	0.378
II	4 (80.0)	5 (55.6)	1.659	0.238
III	0 (0.0)	0 (0.0)	-	-
Molecular typing				
Luminal	4 (80.0)	5 (55.6)	0.873	0.378
Non-Luminal	1 (20.0)	4 (44.4)	0.873	0.378
blood routine test				
WBC ($\times 10^9/L$)	5.4 (5.0, 6.6)	5.7 (4.6, 7.2)	-0.244	0.807
ANC ($\times 10^9/L$)	3.6 (3.0, 4.3)	3.4 (2.9, 5.5)	-0.244	0.807
HBG (g/L)	120.0 (116.8, 128.0)	126.0 (118.0, 144.0)	-0.293	0.770
PLT ($\times 10^9/L$)	223.0 (199.0, 270.0)	252.0 (195.0, 288.0)	-1.126	0.260

Table 3. Demographic Characteristics and Baseline Measurements of verification group (N=17).

Characteristic	IT (N=10)	OC (N=7)	t/ χ^2/Z	P- value
Age	26~75 (54.5±10.1)	33~70 (55.1±9.7)	-0.276	0.783
Menstrual status				
Premenopausal	2 (20.0)	2 (28.6)	0.168	0.559
Postmenopausal	8 (80.0)	5 (71.4)	0.168	0.559
Chemotherapy				
Anthracyclines only	0 (0.0)	0 (0.0)	-	-
Taxanes only	4 (10.0)	1 (14.3)	0.565	0.441
Anthracycline combined with taxanes	6 (60.0)	6 (85.7)	1.311	0.338
Tumor stages				
I	3 (30.0)	5 (71.4)	2.837	0.117
II	7 (70.0)	1 (14.3)	0.565	0.441
III	0 (0)	1 (14.3)	1.518	0.412
Molecular typing				
Luminal	9 (90.0)	6 (85.7)	0.073	0.669
Non-Luminal	1 (10.0)	1 (14.3)	0.073	0.669
blood routine test				
WBC ($\times 10^9/L$)	5.9 (4.4, 7.0)	5.8 (4.8, 7.4)	-0.200	0.841
ANC ($\times 10^9/L$)	3.4 (2.8, 4.3)	4.2 (3.0, 5.2)	-0.869	0.385
HBG (g/L)	124.0 (110.5~ 127.5)	134.0 (116.5~ 141.0)	-0.067	0.947
PLT ($\times 10^9/L$)	230.0 (203.5~ 265.5)	261.0 (164.5~ 304.0)	-1.403	0.161

Figures

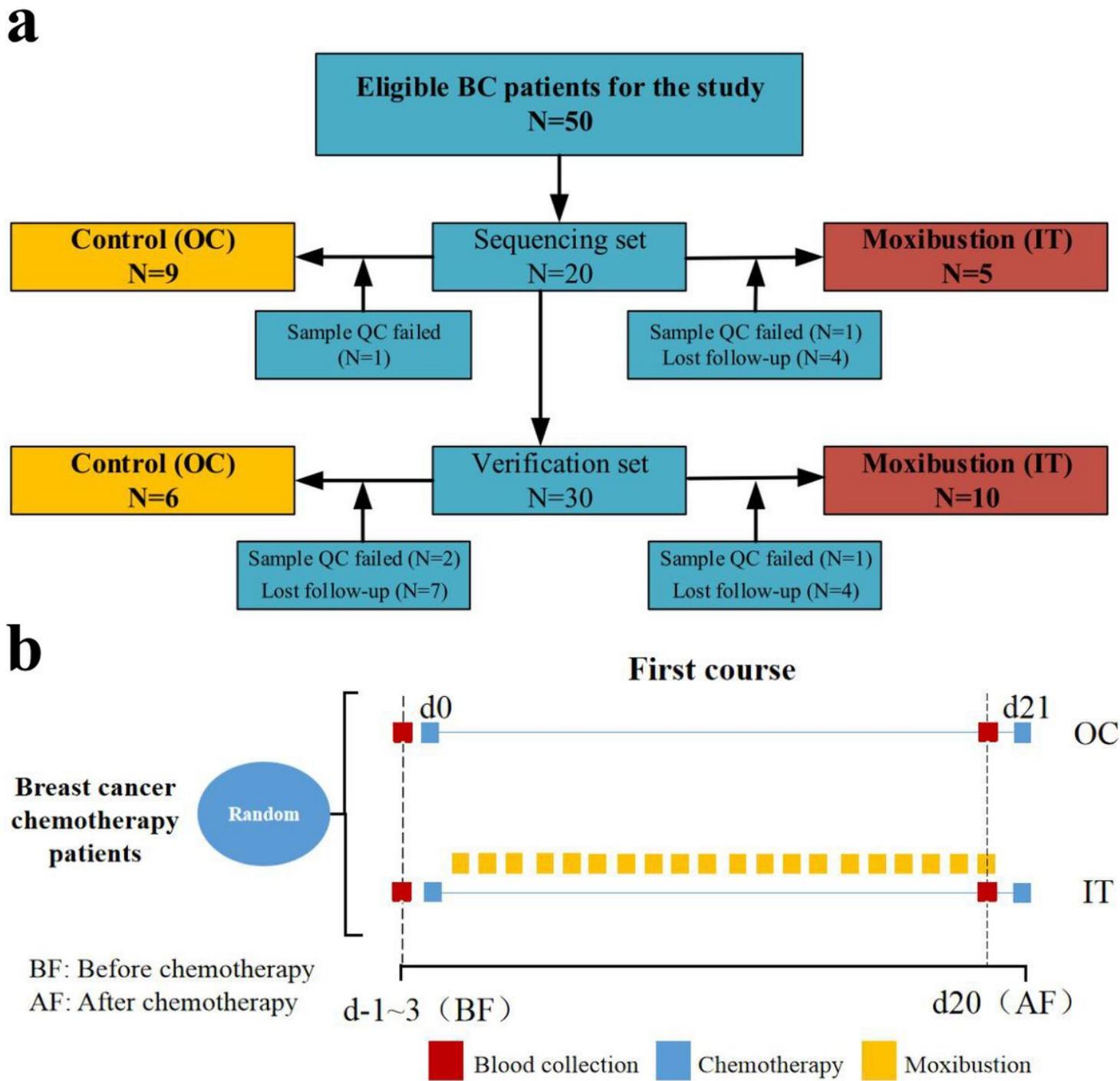


Figure 1

Flow chart of patient and sample allocation.

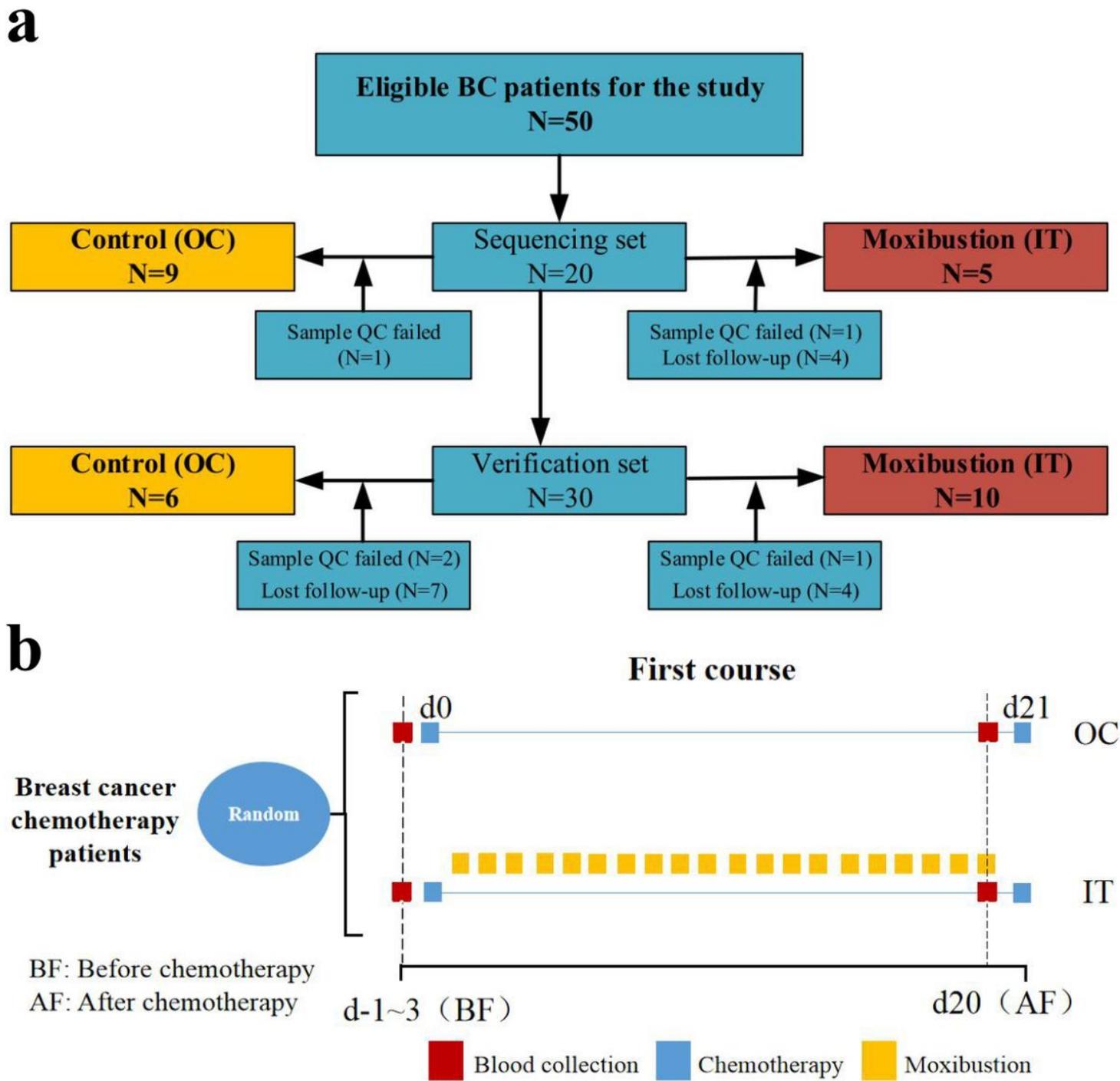


Figure 1

Flow chart of patient and sample allocation.

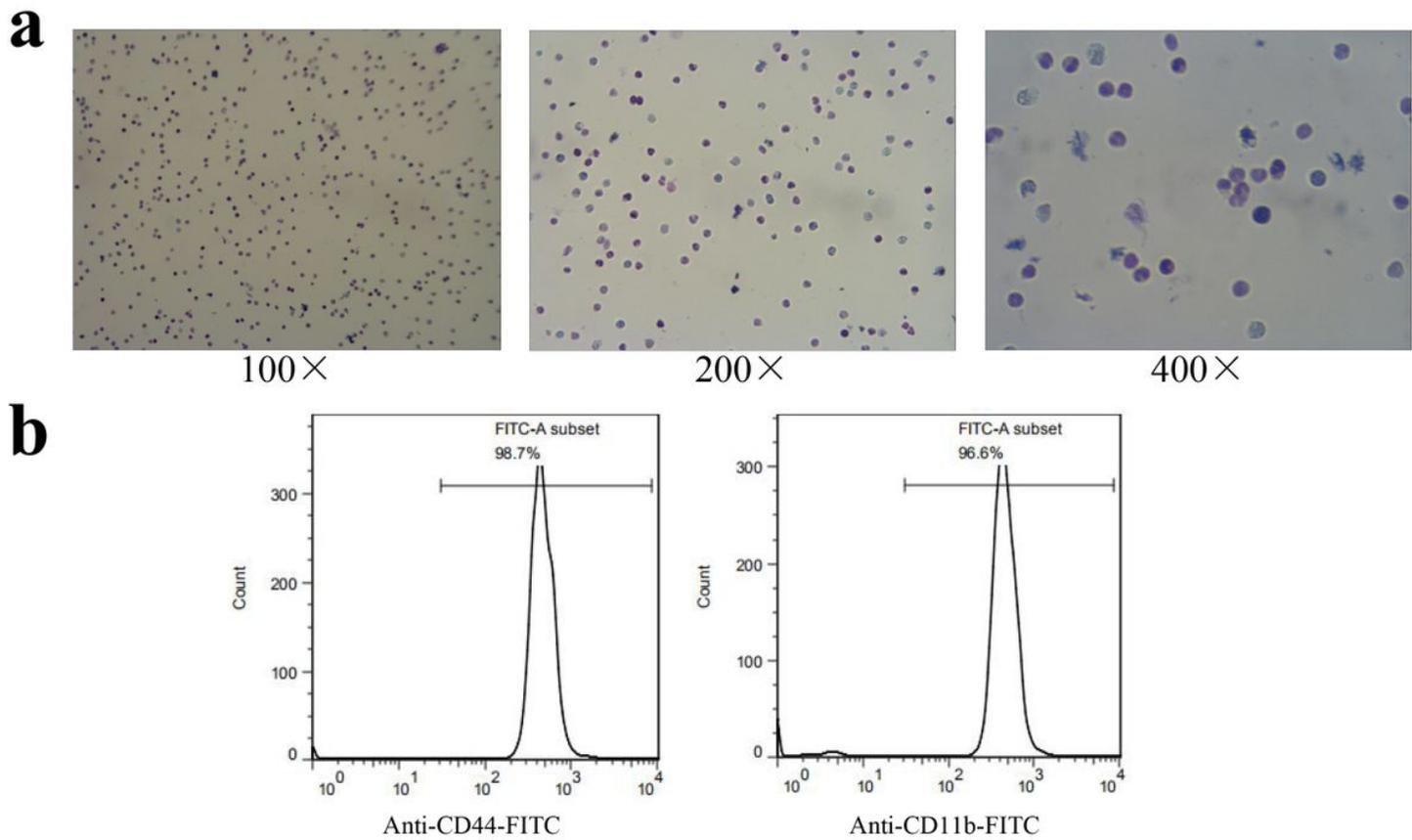


Figure 2

Neutrophils identified by Diff-quick staining and flow cytometry.

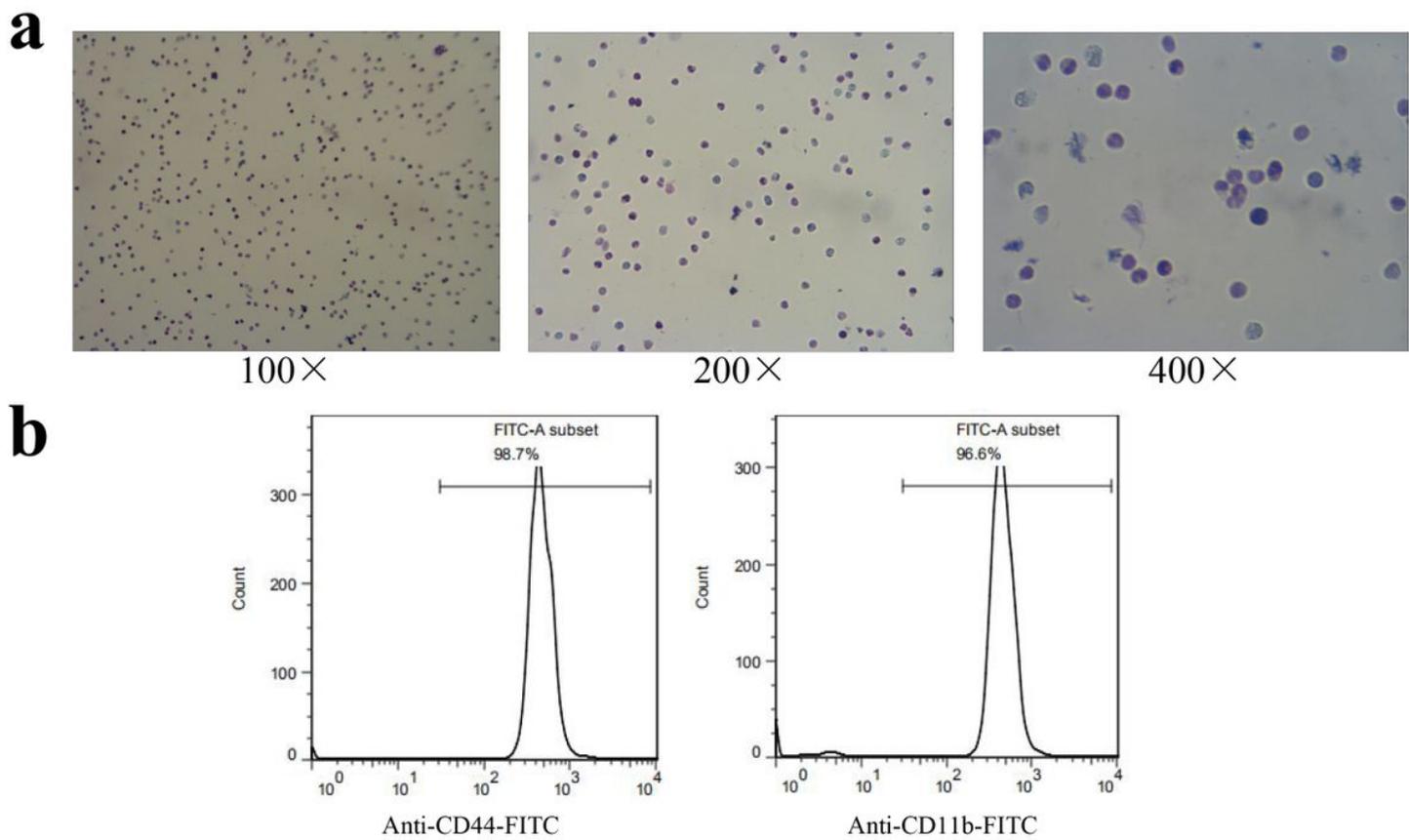


Figure 2

Neutrophils identified by Diff-quick staining and flow cytometry.

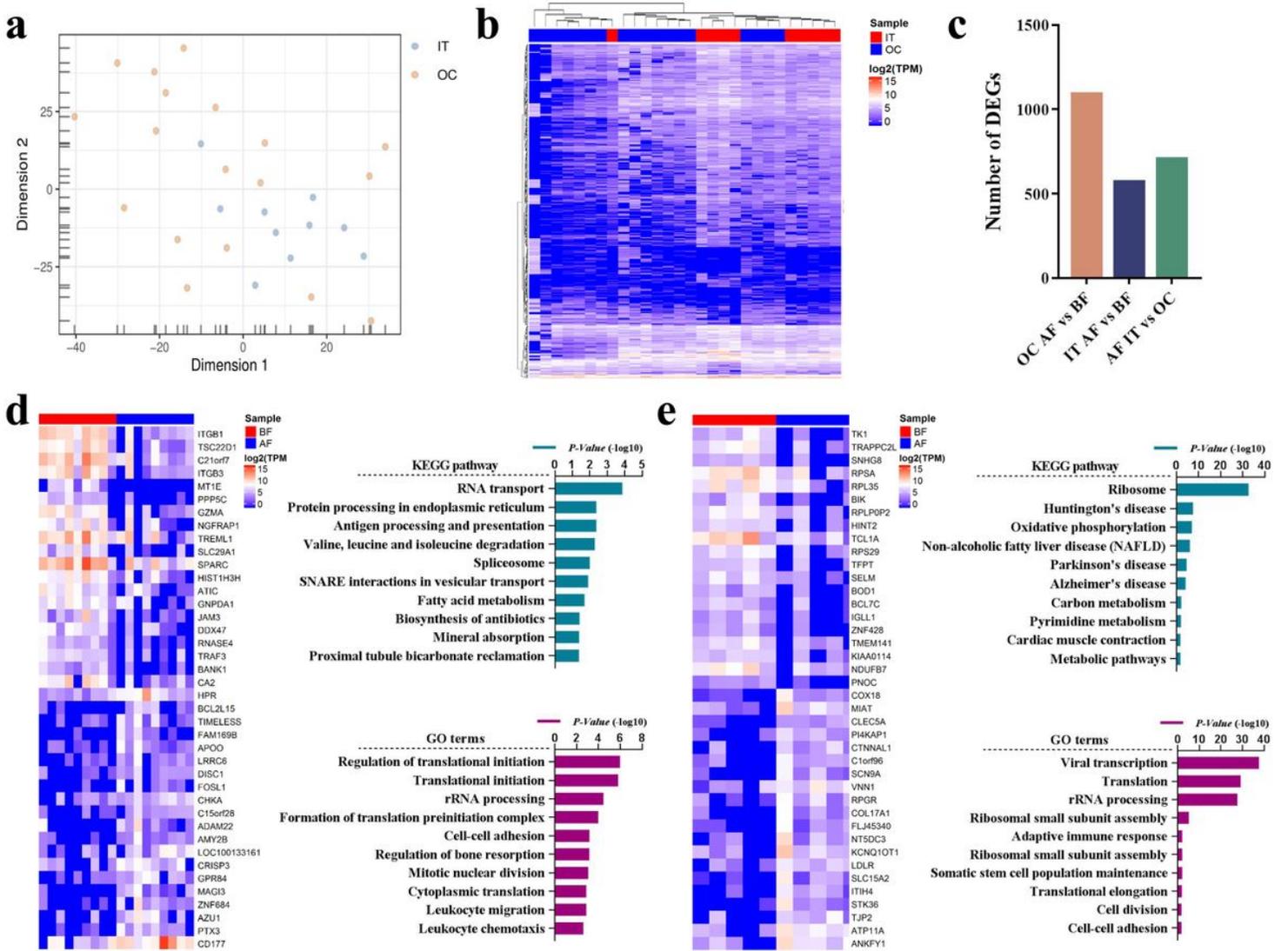


Figure 3

(a, b). PCA and HCA. (c). Number of DEGs identified in OC_AF vs. OC_BF, IT_AF vs. IT_BF, and OC_AF vs. IT_AF groups. (d). The top DEGs and biological function analyses of DEGs in the OC_AF vs. OC_BF groups. (e). The top DEGs and biological functions analyses of DEGs in the IT_AF vs. IT_BF groups.

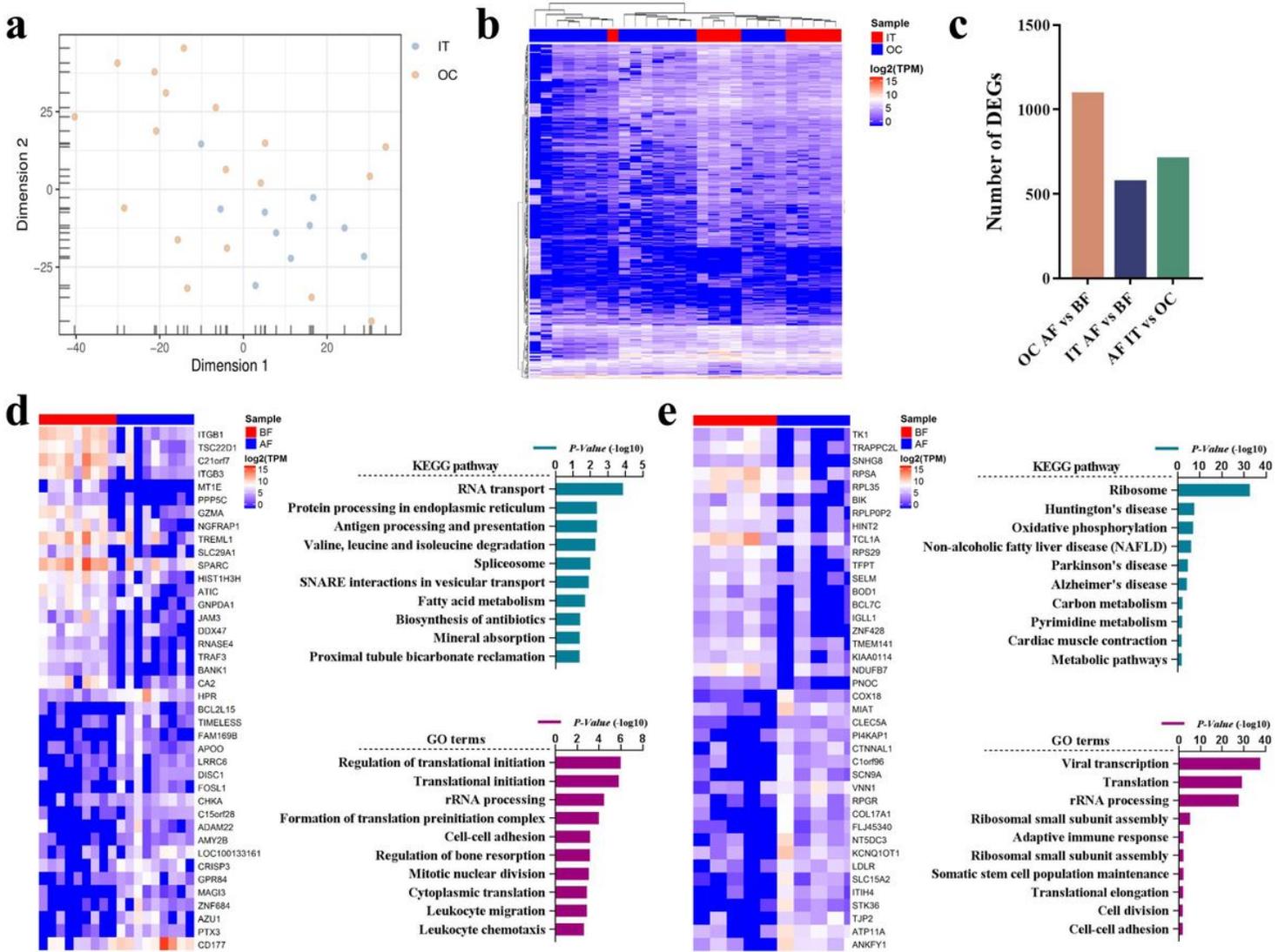


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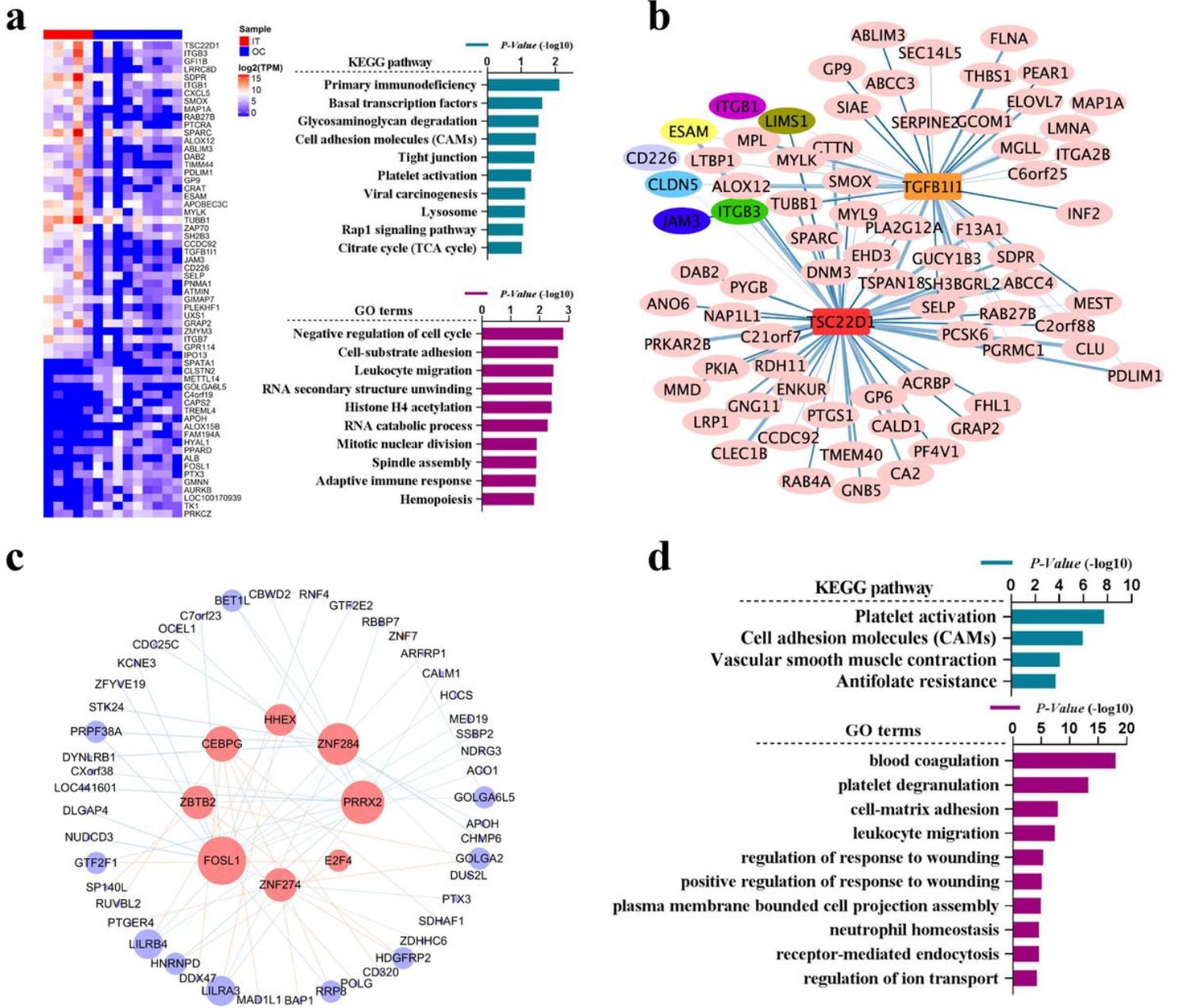


Figure 4

(a). The top DEGs and biological functions analyses of DEGs in the OC_AF vs. IT_AF groups. (b). Co-network of top DEGs in the OC_AF vs. IT_AF groups. (c). Nuclear TF regulatory network. (d). KEGG and GO analyses of key genes in the nuclear TF regulatory network.

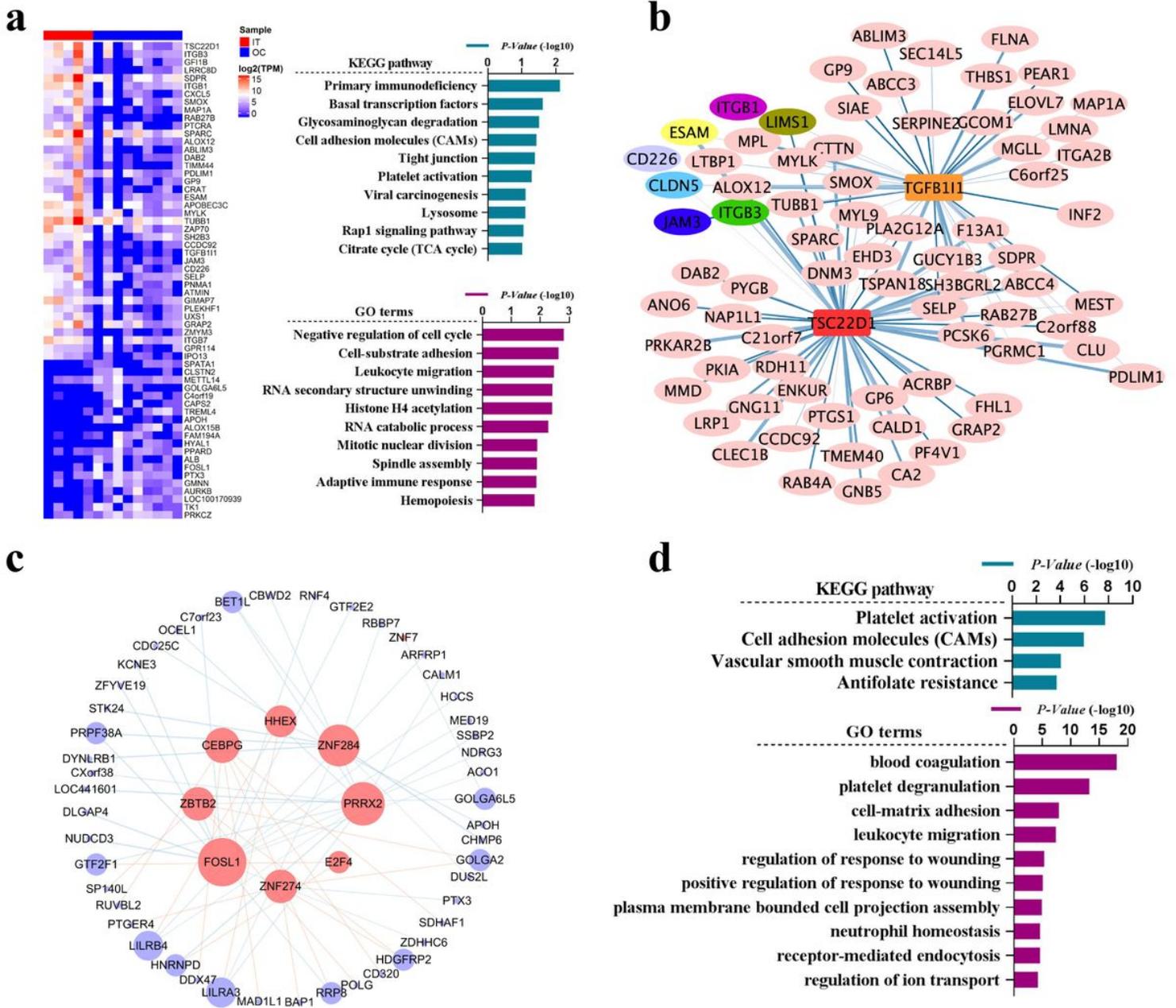


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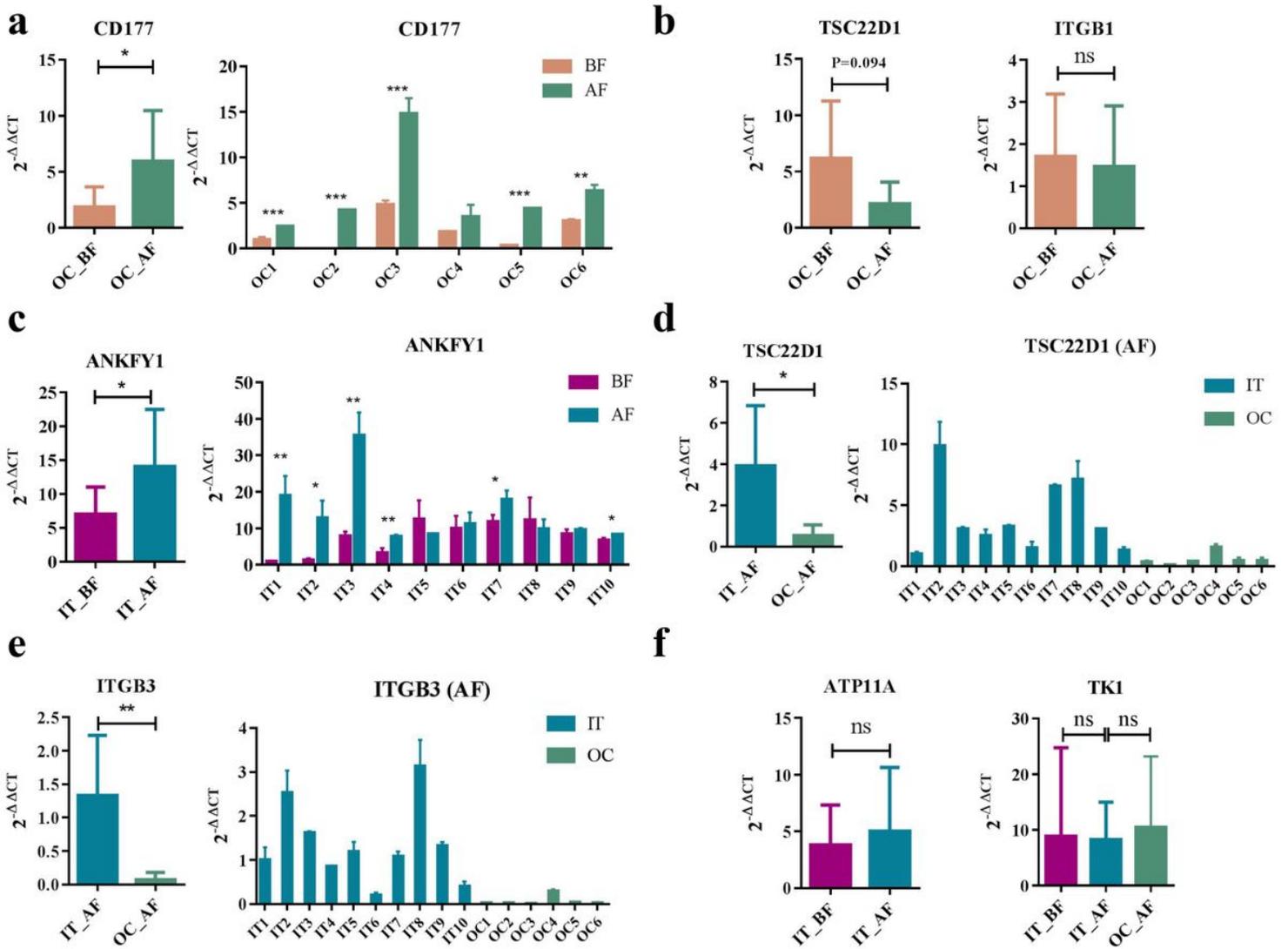


Figure 5

(a). CD177 mRNA expression in the OC_AF vs. OC_BF groups and in each patient in the OC group. (b). TSC22D and ITGB1 mRNA expression in the OC_AF vs. OC_BF groups. (c). ANKFY1 mRNA expression in the IT_AF vs. IT_BF groups and in each patient in the IT group. (d, e). ITGB3 and TSC22D1 mRNA expression in the OC_AF vs. IT_AF groups and in each patient in the AF group. (f). ATP11A and TK1 mRNA expression in each group.

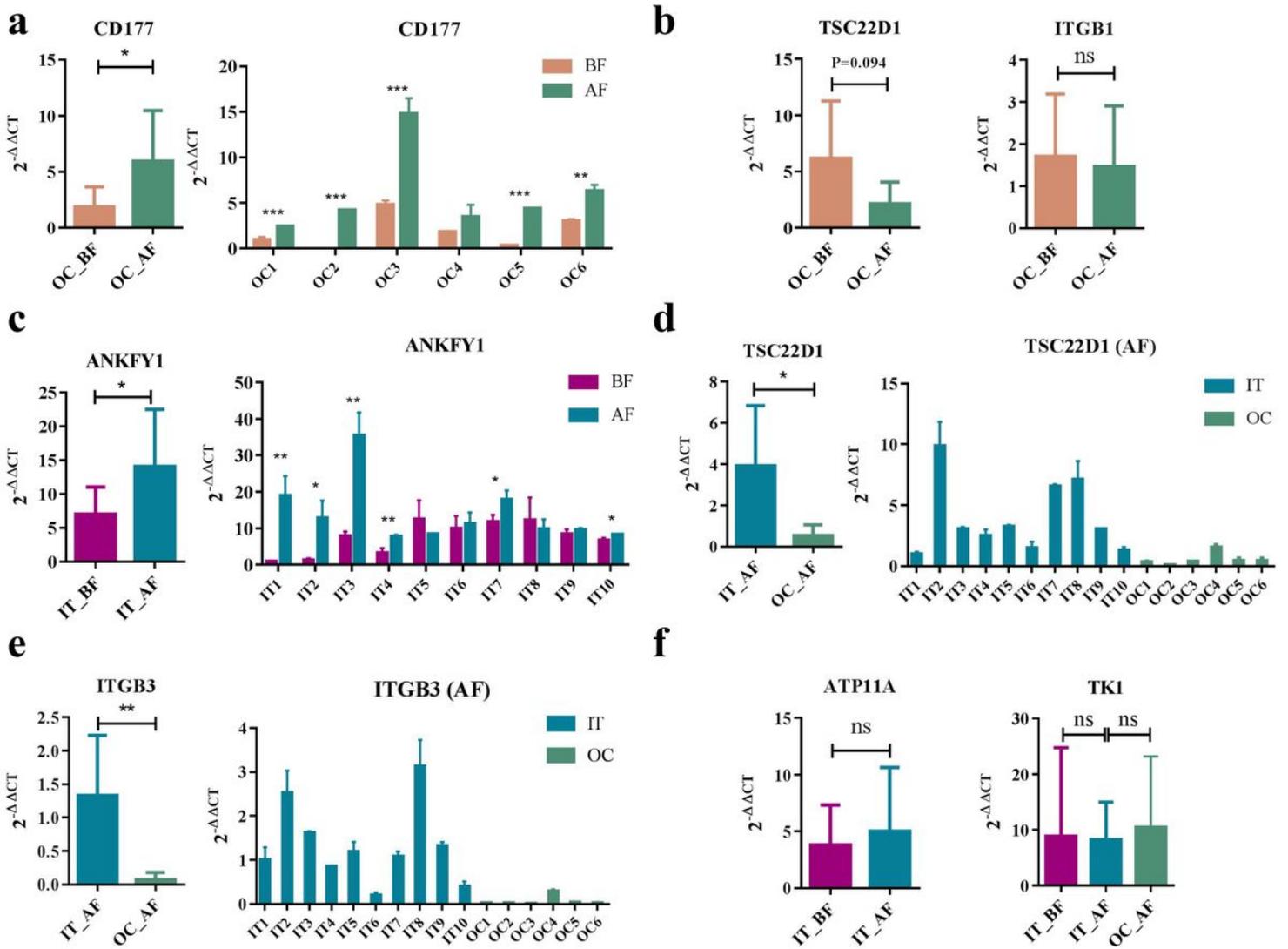


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(a). CD177 mRNA expression in the OC_AF vs. OC_BF groups and in each patient in the OC group. (b). TSC22D and ITGB1 mRNA expression in the OC_AF vs. OC_BF groups. (c). ANKFY1 mRNA expression in the IT_AF vs. IT_BF groups and in each patient in the IT group. (d, e). ITGB3 and TSC22D1 mRNA expression in the OC_AF vs. IT_AF groups and in each patient in the AF group. (f). ATP11A and TK1 mRNA expression in each group.