Study on the Role of Calreticulin Within Platelet from Adult Patients with Chronic Immune Thrombocytopenic Purpura

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Abstract To observe the differences in proteins between adult patients with chronic immune thrombocytopenic purpura (ITP) and healthy adults. 30 patients with chronic ITP and 30 healthy controls were enrolled into the study. The platelet total protein was extracted from peripheral venous blood of 10 chronic ITP patients and 10 healthy controls respectively, and subjected to two-dimensional electrophoresis (2-DE) to find the differential protein spot between chronic ITP patients and healthy controls, then the differential protein spots were identified by mass spectrometry. Subsequently, platelets RNA and proteins were isolated from the other 20 chronic ITP patients and 20 healthy controls respectively, and used for confirming the 2-DE and mass spectrometry results by using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and enzyme linked immunosorbent assay (ELISA).

2-DE combined with mass spectrometry revealed that calreticulin (CRT) expressed normally within platelets from healthy controls, while it reduced within platelets from patients with chronic ITP. qPCR and ELISA confirmed that CRT was decreased at both RNA transcription and protein expression levels within platelets from chronic ITP patients compared with healthy controls. Decreased transcription and expression of CRT within platelets may play an important role in the pathogenesis of chronic ITP, which is worthy of further study.

Keywords Chronic immune thrombocytopenic purpura (cITP) · Platelets · Calreticulin (CRT) · Two-dimensional electrophoresis (2-DE) · Mass spectrometry · Silver staining · Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) · Enzyme linked immunosorbent assay (ELISA)

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Introduction

ITP is a common hemorrhagic disorder, accounting for about 30% of bleeding disorders, in which enhanced platelet destruction and weakened platelet production lead to thrombocytopenia and thus mucocutaneous bleeding. ITP is divided into two types including acute and chronic in clinic, in which the former attacks usually children and youngsters and is often self-limiting, but the latter annoys usually adults, especially young women, and is a chronic and life-long condition. Up to now, the pathophysiology of ITP has been extensively investigated. It is generally accepted that a complex multifactorial immune dysregulation and dys-proliferation of platelets account for the primary mechanism, while proliferation and maturation disorders of megakaryocytes are also involved [1].

Nevertheless, after treatment with anti-immune and thrombopoietic strategy, most patients with acute ITP (aITP) can be cured, while patients with chronic ITP (cITP) often show transient increment of platelet count followed by recurrence once the therapy is reduced or discontinued. Thus, the underlying pathogenic events leading to chronic ITP remain elusive. Whether there are some abnormalities within platelets themselves from patients with chronic ITP or not? What are the differences between platelets from patients with chronic ITP and healthy individuals? In order to answer these questions and to explore the pathophysiology of chronic ITP on the level of platelet intracellular proteins, we investigated the differential proteins that existed within platelets from adult patients with chronic ITP. We identified the differential protein spot of platelets between the cITP patients and healthy individuals by using two-dimensional electrophoresis (2-DE) and mass spectrometry, and confirmed afterwards by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and enzyme linked immunosorbent assay (ELISA) respectively.

Materials and Methods

Patient Enrollment

Patients who were admitted to the Division of Hematology, the Second Affiliated Hospital of Shantou University Medical College, were diagnosed for chronic ITP according to the guidelines and criteria from the International Working Group. 30 adult female patients aged from 23 to 54 years old, diagnosed as having chronic primary ITP, which manifested with persistent thrombocytopenia ($< 100 \times 10^9/L$) for at least 12 months, along with the absence of any other disease that can cause thrombocytopenia were included in the study. No therapeutic measure was administrated on these chronic ITP patients within at least 3 weeks. We also enrolled 30 healthy volunteers (female, control) from the Health Examination Center, the Second Affiliated Hospital of Shantou University Medical College, who presented normal platelet count [$(213.9 \pm 54.2) \times 10^9/L$] and no concurrent illnesses or medications at the time of blood draw. Those volunteers were matched with chronic ITP patients according to age and gender (female). There were no statistical differences in age, counts of white blood cell (WBC), red blood cell (RBC) and hemoglobin (HGB) between cITP and control group. The study protocols were conducted according to the principles of the Declaration of Helsinki, and were approved by the Scientific and Medical Ethical Committee of the Second Affiliated Hospital of Shantou University Medical College. All the subjects gave their written

informed consent (appended at the last page) before their enrollment in the study. 30 chronic ITP patients and 30 healthy controls were divided equally into 3 subgroups respectively, and used for 2-DE/mass spectrometry, qPCR and ELISA analysis separately (Tables 1, 2, 3).

Methods

Platelet Isolation

Venous blood (6 mL) was collected from each patient or healthy control into dipotassium ethylene diamine tetraacetate (EDTA-2 K, 5 mM, final concentration) and prostaglandin E1 (PGE1, 100 nM, final concentration). After centrifugation (10 min at 200g), the top two-thirds of platelet-rich plasma (PRP) was then spun at $1000 \times g$ for 10 min and the supernatant was discarded, the tube containing pellet was inverted on a sterile tissue for 1 min and then wipe out their walls from supernatant's remnants. The pellet containing platelets was then re-suspended in 1.0 ml of modified Tyrode's buffer [10 mM 4-(2-hydroxyethyl) piperazine-1-erhaesulfonic acid (Hepes), 138 mM NaCl, 2.7 mM, 0.4 mM NaH_2PO_4 , 10 mM NaHCO_3 , 5 mM glucose, 100 nM PGE1, pH7.4] and allowed to sit for 30 min. Platelets were additionally centrifuged (200g for 5 min) to remove residual leukocytes. The supernatants were centrifuged (1000g for 10 min), and finally resuspended in the same buffer. Centrifugations were done at room temperature (RT). Leukocyte contamination was evaluated by microscopy using Wright's staining. Cell counts were performed by taking 10 μl of platelets suspension, diluting it using the Unopette collection system (Becton–Dickinson, Franklin Lakes, NJ, 1:100 dilution) and counting the cells with a hemocytometer. Leukocyte counts were always less than $1/10^5$ platelets.

Two-Dimensional Gel Electrophoresis (2-DE) [2]

Extraction of Platelets Protein and Quantification For 2-DE and mass spectrometry analysis, platelets isolated from 10 chronic ITP patients were mixed together to constitute a mixed test sample cell, while those from 10 healthy controls was also mixed together to constitute a mixed control sample cell. Tested and control sample cells were washed with phosphate buffer saline (PBS) and lysed in a lysis buffer [8 M urea, 30 mM Hepes, 0.5% sodium dodecyl sulfate (SDS), 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM EDTA-2 K, and 10 mM DL-dithiothreitol (DTT)]. The solution was dispersed by sonication for 5 min (power 180 W, pulse 2 s on and 3 s off), then centrifuged at $20,000g$ for 30 min. The supernatant was collected and protein concentration was determined by the Bradford protein assay kit (Bio-Rad Laboratories,

Table 1 Basic characteristics of chronic ITP patients and healthy controls for 2-DE and mass spectrometry

No.	Age (year)	WBC ($\times 10^9/L$)	RBC ($\times 10^{12}/L$)	HGB (g/L)	PLT ($\times 10^9/L$)
Chronic ITP patients					
1	43	6.28	4.62	117	20
2	40	7.15	4.75	145	15
3	34	5.42	4.68	128	9
5	23	7.59	5.11	109	4
6	47	4.87	4.29	121	19
7	41	9.17	5.25	118	26
8	32	4.48	4.84	110	30
9	34	7.32	4.07	113	35
9	54	9.06	4.07	131	29
10	37	4.37	4.76	125	12
Healthy controls					
1	23	4.03	4.38	118	256
2	43	5.63	4.35	131	197
3	37	7.71	5.05	144	363
4	33	5.02	4.26	114	223
5	45	8.20	4.27	130	249
6	44	5.45	4.58	121	260
7	34	5.29	4.82	143	208
8	36	4.05	4.37	134	148
9	35	6.40	4.11	125	159
10	53	4.48	4.83	124	149

Table 2 Basic characteristics of chronic ITP patients and healthy controls for RT-qPCR

No.	Age (year)	WBC ($\times 10^9/L$)	RBC ($\times 10^{12}/L$)	HGB (g/L)	PLT ($\times 10^9/L$)
Chronic ITP patients					
1	31	5.65	4.45	134	53
2	54	8.87	3.98	141	41
3	27	4.21	4.61	117	7
4	38	5.76	5.02	129	30
5	25	7.58	4.14	108	22
6	35	7.28	4.87	133	37
7	30	6.09	4.30	121	15
8	44	5.11	4.75	140	36
9	46	4.95	4.22	123	44
10	43	7.29	4.84	136	25
Healthy controls					
1	29	4.16	4.71	133	291
2	37	4.77	4.76	117	209
3	24	5.89	4.33	120	274
4	36	9.26	4.25	114	183
5	31	7.58	4.19	140	206
6	41	7.73	4.23	134	272
7	47	5.91	4.72	109	215
8	54	5.44	4.88	115	233
9	42	8.40	4.16	132	111
10	30	4.95	4.77	126	128

Hercules, CA, USA) using bovine serum albumin as a standard [3].

Table 3 Basic characteristics of chronic ITP patients and healthy controls for ELISA

No.	Age (year)	WBC ($\times 10^9/L$)	RBC ($\times 10^{12}/L$)	HGB (g/L)	PLT ($\times 10^9/L$)
Chronic ITP patients					
1	34	8.74	4.93	127	45
2	44	7.77	4.67	114	61
3	43	4.16	4.11	124	11
4	51	5.57	4.85	126	38
5	38	8.03	4.33	133	29
6	52	6.23	4.19	112	33
7	49	5.68	4.57	112	17
8	23	6.38	4.19	110	10
9	24	7.18	3.96	129	26
10	25	6.06	4.35	111	14
Healthy controls					
1	24	4.95	4.20	126	174
2	26	5.85	4.16	114	191
3	47	7.78	4.74	125	188
4	27	4.63	4.23	141	251
5	34	6.34	4.62	137	166
6	37	7.01	4.44	113	241
7	45	5.42	4.50	127	285
8	35	9.03	4.85	116	184
9	50	5.53	4.57	134	205
10	46	4.94	4.73	120	199

Pre-electrophoresis Gel solution [Urea (12 M), 1% (volume fraction) of Triton X-100, acrylamide (1 M), 6.5% amphoteric electrolyte (pH 5–7), 3.2% amphoteric electrolyte (pH 3–9.5), 8% (volume fraction) glycerol, ammonium persulfate (5 mM), 0.2 μ L TEMED] was poured into 2 clean glass tubes with 1 mm diameters, which were placed with a horizontal angle of 100°. After the gel polymerization, it was immobilized on the first-dimension inner chamber of the electrophoresis tank. 400 ml NaOH (0.8 M) solution was added to the top of the chamber, and 4000 mL phosphoric acid (10 mM) solution was added outside the inner chamber respectively. Pre-electrophoresis was performed using a voltage of 200 V for 15 min, 300 V for 15 min, 400 V for 75 min.

First-Dimension Isoelectric Focusing (IEF) Electrophoresis After pre-electrophoresis, tubes were taken out and 10 μ L platelet total protein (3–4 μ g/L) and 2 μ L bromophenol blue were added into tubes and were covered with NaOH solution. Then tubes were assembled to the first-dimension inner chamber of the electrophoresis tank. 400 ml of NaOH (0.8 M) electrophoresis solution was added to the top of the chamber and the first-dimension Isoelectric focusing (IEF) electrophoresis (the pH gradient: 3–7) was performed using a voltage of 500 V for 20 h.

Equilibration The gel in tubes was added into the strip equilibration buffer I [0.2 g dithiothreitol was dissolved in a strip equilibrated mother liquor [10 mL, 6 M Urea, 50 mM Tris–HCl, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.03 mM bromophenol blue] and the strip equilibration buffer II [0.3 g iodoacetamide was dissolved in another strip of equilibrated mother liquor (10 mL)] respectively, and equilibration was done for 15 min.

The Second-Dimension Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) Ingredients for 10% (mass fraction) of resolving gel solution was 1.5 M acrylamide, 378 mM Tris–HCl (pH8.8), 0.1% (mass fraction) SDS, 0.1% (mass fraction) ammonium persulfate and 0.04% (volume fraction) TEMED. Ingredients for 5% stacking gel solution was 0.7 M Acrylamide, 0.2 M Tris–HCl (pH8.8), 0.1% SDS, 0.1% ammonium persulfate and 0.1% TEMED. The ingredients of each gel solution was added in the designated solution bottle and mixed with pump. After mixing, the gel solution was deaerated for 15 min with the vacuum pump. The solution bottle was again connected to the pump for gel casting process. After casting and gel polymerization, the equilibrium strip was placed at the top of the stacking gel, covering the low melting point agarose sealant. Then the clean glass plate was fixed to the second-dimension inner chamber of the

electrophoresis tank, and tris–glycine electrophoresis buffer was added.

Electrophoresis was performed using a voltage of 90 V. When the dye indicator was in a straight line (the dye front), the voltage was changed to 150 V until the dye front reached 2.0 cm from the gel bottom. All gels were stained with bromophenol blue and destained with several changes of water. The protein in each sample cell was repeated twice in 2-DE.

Silver Staining After electrophoresis, separation gel was taken out and silver staining was performed [4], to find out the differential protein spot (Abundance difference threshold > 2.0 was considered as significant difference [5]) by using PD quest software (Bio-Rad Laboratories, Inc. USA). Later, the differential protein spot was cut and mass spectrometry was performed at Shanghai Bo-Yuan Biological Technology Co., Ltd. China.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA of platelets from another 10 chronic ITP patients and 10 healthy controls matched by age and gender were prepared with Trizol reagent (Life Technologies, Carlsbad, CA, USA). cDNA samples were obtained by PrimeScript™ RT reagent Kit (cat. no. RR037A, Takara Bio, Inc., Dalian, China). Real time PCR quantification for CRT [primer sequences were 5'-CTGGCACCATCTTT-GACAACT-3' (forward), and 5'-TTTGTACGCCC-CACGTCT-3' (reverse)] was done in triplicate, in a 20 µL reaction final volume, from 2 µL of cDNA, in a CFX96 thermocycler (Bio-Rad Laboratories Inc., USA) using SYBR Green PCR kit (cat. no. RR420A, Takara Bio, Inc., Dalian, China) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, primer sequences of which were 5'-TGCAACCACTGCTTAGC-3' (forward), and 5'-GGCATGGACTGTGGTCATGAG -3' (reverse) [6]. The fold change of mRNA was calculated by use of the comparative Ct method.

Enzyme Linked Immunosorbent Assay (ELISA)

Total protein from platelets was isolated and quantified as described above, which were collected from another 10 chronic ITP patients and 10 healthy controls matched by age and gender. ELISA kit for human calreticulin (cat. no. DEIA6937; Creative Diagnostics, Shirley, NY, USA) was utilized to validate its levels in chronic ITP patients and healthy controls according to the instructions provided by the manufacturers. Absorbance was determined using PerkinElmer EnSpire Multimode Plate Reader 2300

(PerkinElmer, Inc., MA, USA) and analysis of results was conducted by CurveExpert 1.4 (Daniel Hyams, Starkville, MS, USA).

Statistical Analysis

Data for RT-qPCR and ELISA are expressed as mean ± SD. A two-tailed Student's *t* test was used to determine the differences between chronic ITP patients and healthy controls. Statistical analyses were performed using the Statistical Program for Social Sciences (SPSS) 20.0 software (SPSS Inc. Chicago, IL, USA). A *p* value < 0.05 was considered statistically significant.

Results

2-DE and Silver Staining of Platelet Total Protein

2-DE and silver staining revealed that there was an enhanced protein spot from platelets of healthy controls compared with chronic ITP patients (Fig. 1, indicated by the arrow).

Fig. 1 2-DE and silver staining of platelet total protein from chronic ITP patients and healthy controls. An enhanced protein spot was present in healthy controls compared with chronic ITP patients

Mass Spectrometry and Protein Spot Identification

The protein strip of the differential spot after 2-DE was cut and through enzymatic hydrolysis and matrix-assisted laser desorption ionization (MALDI) successively, the differential protein spot between healthy controls and patients with chronic ITP was broken down into peptide fragments. Figure 2 shows the secondary mass spectrum after mass spectrometry of the peptide segments. Based on mass spectrometry results, information about the fragments were retrieved using Protein Data Bank and identified by using Basic Local Alignment Search Tool (BLAST). Eventually, the protein spot was identified as calreticulin (CRT) (Table 4).

Validation of the 2-DE and Mass Spectrometry Data by RT-qPCR

To confirm the result of 2-DE and mass spectrometry analysis, RT-qPCR was performed to detect the transcription level of CRT. The result showed that CRT was downregulated (Fig. 3) in chronic ITP patients. Which was consistent with the result of 2-DE and mass spectrometry analysis.

Validation of the 2-DE and Mass Spectrometry Data by ELISA

Quantitation by using ELISA found that the contents of CRT within platelets were less from chronic ITP patients than those from healthy controls ($p < 0.01$), which indicated that the alteration of CRT expression was consistent with the 2-DE and mass spectrometry results (Table 5).

Discussion

CRT in humans is encoded by the CALR gene, which is located on chromosome 19 (p13.2–p13.3). The gene is 3.6 kb consisting of 9 exons and 8 introns [7]. The coding

DNA is 1254 bp length, which codes an acidic protein consisting of 417 amino acids. CRT has a highly negative charge ($pI = 4.7$) and a molecular weight of 46.6 kDa, which consists of three structurally and functionally distinct domains, called N, P, and C-terminal domain respectively [7].

N-domain encompasses approximately the first 200 amino acid residues and is the most conserved region of the CALR gene. Structurally, it is a globular domain with 8 antiparallel β -sheets and a helix-turn-helix motif at its N-terminus. Functionally, it is the binding site to integrin and hormone receptors. P-domain is rich in proline residues and encompasses about 30% of residues in the middle of the protein. The domain includes three pairs of repeating sequences that form a paired β -hairpin structure. The domain contains a high-affinity, low capacity Ca^{2+} -binding site. C-terminal domain covers the carboxyl-terminal quarter of the protein and contains a highly acidic C-terminal region (residues 351–359). C-domain is highly negatively charged, and most of residues are aspartic or glutamic acid. Contrary to P-domain, C-domain contains a low-affinity, high-capacity Ca^{2+} -binding site. C-terminus contains a tetra-peptide (KDEL sequence) as an endoplasmic reticulum retention signal and binds multiple calcium ions with low affinity, which is essential for maintenance of cellular calcium homeostasis [7–9].

CRT was initially found as a molecular chaperone, which is a member of the heat shock protein family. CRT is known as an endoplasmic reticulum resident protein 60 (ERp60) [10] and present in all cells other than RBC in higher organisms. In nucleated cells, CRT is not only expressed in endoplasmic reticulum, but also in the nucleus and the cytoplasm. However, CRT is also found at the cell surface of living cancer cells and dying cells, which contributes to the phagocytic uptake of tumor cells and pre-apoptotic cells [11]. Findings of CRT on the surface in many mammalian cells, including some apoptotic and pre-apoptotic cells, tumor cells and platelets, provide clues that this intracellular chaperone protein might function outside the endoplasmic reticulum (ER) [12]. That is to say that non-ER CRT can play an important role in a variety of diverse and important biological processes, for example, tumor and apoptotic cell recognition and wound healing [13]. It is shown that CRT which is a multifunctional Ca^{2+} -binding protein participates in the maintenance of intracellular Ca^{2+} homeostasis, cell adhesion, and chaperoning [14].

As for platelets, non-nucleated cells which are fragments of cytoplasm derived from the megakaryocytes of the bone marrow which enter into the circulation [15], it was shown by Elton et al. [16], using flow cytometry and immunoprecipitation, that CRT is also expressed on the surface of human platelets. Further, CRT existed on the

Fig. 2 Secondary mass spectrum

Table 4 Protein spot identification

Name of protein	Score	Theoretically relative molecular mass	Theoretically isoelectric point	Length
Calreticulin (CRT)	62	48,283	4.23	417 amino acids

Fig. 3 Relative CRT-mRNA level between chronic ITP patients and healthy controls. RT-qPCR showed less expression of CRT at the mRNA level in chronic ITP patients than healthy controls. * $p < 0.05$. Control meant healthy controls, cITP meant chronic ITP**Table 5** Contents of CRT between chronic ITP patients and healthy controls

Content of calreticulin (ng/mg-Pro)	Chronic ITP patients	Controls	<i>p</i> value
	29.251 ± 4.243	572.345 ± 72.364	< 0.001

surface of the membrane and in granulomere, but was not found in the pseudopodia or developed lamellipodia [17].

It is known that when the endothelial layer of vessel is disrupted, collagen and von Willebrand factor (vWF) anchor platelets to the subendothelium. Then platelet GPIb-IX-V receptor binds with vWF and glycoprotein VI (GPVI) receptor and integrin $\alpha 2\beta 1$ bind with collagen [18], following which platelets' adhesion is triggered. CRT can interact with the cell-surface collagen receptors integrin $\alpha 2\beta 1$ and GPVI. Additionally, anti-CRT antibodies can cause platelet activation, inducing Fc gamma RIIa-independent platelet aggregation. It is shown that surface CRT is associated with collagen receptors on the platelet surface, and hypothesized it may play a role in the modulation of the platelet-collagen interaction [16].

CRT has a high affinity with Ca^{2+} , which can relieve overload of intracellular Ca^{2+} by binding with Ca^{2+} . Thereby, the protein can reduce a series of biological responses caused by intracellular Ca^{2+} overload [11], such as mitochondrial dysfunction, activation of Ca^{2+} -dependent degrading enzymes, and promotion of the generation of reactive oxygen species. Resting platelets maintain active calcium efflux via a cyclic AMP activated calcium pump. Intracellular calcium concentration determines platelet activation status. It's known that increasing intracellular Ca^{2+} concentration plays a key role in platelet activation and aggregation [19]. CRT can reduce

intracellular Ca^{2+} concentration because of its high affinity for Ca^{2+} thereby inhibiting platelet activation and aggregation [16]. Additionally, Ca^{2+} overload caused by Ca^{2+} influx promotes the apoptosis of platelets, and CRT can reduce platelet apoptosis by inhibiting Ca^{2+} influx. Thus, we speculate that reduced CRT in platelets of chronic ITP patients accounts for the high Ca^{2+} concentration in platelets, resulting in the state of activation and hyper-apoptosis [20].

In the present study, 2-DE and mass spectrometry identified decreased expression of CRT within platelets from chronic ITP patients, which was confirmed afterwards

by means of RT-qPCR and ELISA respectively. Thus, we hypothesize that thrombocytopenia in ITP may be caused by hyperactivity of apoptosis in platelets because of loss of CRT. On the other hand, it's known that a number of nuclear receptors are present in human platelet, including the receptors for sex steroids, glucocorticoids, and peroxisome proliferator-activated receptors (PPARs) and retinoid X receptors (RXRs) [21]. Many nuclear receptors contain specific homologous sequences to CRT-binding site. CRT can bind to the KLGFFKR synthetic peptide, which is identical to an amino acid sequence in the DNA-binding domain of the superfamily of nuclear receptors. For example, the amino terminus of CRT interacts with the DNA-binding domain of the glucocorticoid receptor and stops the receptor binding to its specific glucocorticoid response element. Hence CRT can regulate the gene transcription of nuclear receptors. We infer that CRT, as a signal transduction molecule, binds to both Ca^{2+} and nuclear receptors of platelets, thereby exerting extensive biological activity. Platelets of chronic ITP patients lack CRT and so the gene transcription is disturbed, which may be one of pathogenesis for thrombocytopenia in ITP.

In summary, both mRNA transcription and protein expression of CRT were decreased within platelets from chronic ITP patients, which is an important biological feature of platelets from chronic ITP patients. While, much remains to be understood about the roles of CRT in

platelets, further studies are required for clarification in this area.

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Compliance with Ethical Standards

Ethical Approval This study was approved by the ethical review committee of the Second Affiliated Hospital of Shantou University Medical College.

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