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College of Medicine
Baghdad, Iraq
Tel and Fax: + 964-1-5224368
P. O. Box 14222, Baghdad, Iraq.
E-mail: iraqijms@colmed-alnahrain.edu.iq

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Iraqi Journal of Medical Sciences

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Kidney Transplantation from Brain Dead Donors: Why and Where Do We Stand?

Ausama S. Abdul Muhsin *FIBMS, FEBU*

Section of Urology, Dept. of Surgery, College of Medicine, Al-Nahrain University, Baghdad, Iraq.

Abstract

Living organ donation is the most widely practiced type of donation in the Middle East and includes kidney and partial liver. It is predominantly genetically related, however, non-genetically related and commercial living organ donation do exist. Other sources of organ donation include organs obtained from a donor after brain stem death (BD) also called (cadaveric heart beating donors), or donation after cardiac death (DCD) previously known as non – heart beating donation.

The objectives of this paper are to explain the rationale of using organs from BD donors, highlight the concept of BD, show legal, religious and ethical related issues and demonstrate the international experience and status of BD organ (kidney) transplantation in Iraq.

Introduction

Chronic kidney disease (CKD) is a common and costly health problem in the Middle East. Hemodialysis (HD) is still the major modality of renal replacement therapy (RRT) in the Middle East [(1)]. In Iraq HD is almost the only type of chronic dialysis. There are no data available on adequacy of HD in our country. The mean duration of Iraqi patients on HD in one study was shown to be about 26 months, while it is 82 months in Jordan study [(2)], whether this is due to an excess mortality among our CKD patients, loss of follow up due to inadequate clinical reporting system, or other factors yet to be determined.

The ideal treatment for end-stage renal disease (ESRD) is kidney transplantation (KT). However, the considerable shortage of donor organs and the increasing number of patients with ESRD on KT waiting lists often have resulted in unacceptably long waiting times for an appropriate organ allograft [(3)]. Other sources of organ donation include organs obtained from a donor after brain stem death (BD) also called (cadaveric heart beating donors) after BD criteria were defined and adopted in 1968, or donation after cardiac death (DCD) previously known as non – heart beating donation [(4,5)] or even ABO – incompatible living donor kidney transplantation [(3)]. Unfortunately paid living – unrelated (commercial) kidney transplantation or sometimes called (transplantation tourism) does exist and it is not only controversial for ethical aspects, but has been reported to result in serious complications in the postoperative period that cause high rates of morbidity and mortality, and it also carries the risk of a negative effect on local transplant programs [(6)].

Clinical issues of BD organ donation

Death can be considered in terms of medical, legal, ethical, philosophical, societal, cultural, and religious rationales. The medical definition of death is primarily a scientific issue based on

the best available evidence. There is growing consensus that there is a unifying medical concept of death; all human death is anatomically located to the brain. That is, human death involves the irreversible loss of the capacity for consciousness, combined with the irreversible loss of the capacity to breathe. These two essential capacities are found in the brain, particularly the brainstem, and represent the most basic manner in which the human organism can sense and interact with its environment [7, 8]. In other words BD is an irreversible status and can be considered as death.

Organs can be obtained from BD potential donors **providing that** the diagnosis of BD is achieved by a committee of expert clinical personnel, the patient is maintained on ventilator with intact circulation (usually in an intensive care unit ICU), there is/are no clinical contraindication(s) for organ retrieval such as HIV infection, current neoplastic disease …etc, and obtaining a consent for organ donation either from the deceased person (prior to his/her death for e.g. having a donor card), the family/ next of kin and sometimes the coroner and designated officer of the hospital [9] . The diagnosis of BD and the following steps required for organ retrieval and then transplantation are beyond the scope of this paper. An excellent BD organ donation program for e.g. that of United Network for Organ Sharing (UNOS) in USA allows single and multiple organ transplantation such as (Kidney – Pancreas), (Kidney – Heart – Lung), (Kidney – Pancreas – Heart), …etc and more than one patient may benefit from a single BD donor [10].

Religious issues

In 1986, the Islamic theologians (Al Aloma) issued what became known as the Amman declaration, in which they clearly accepted BD and the retrieval and transplantation of organs from living and cadaveric donors. The main consideration for the Islamic scholars Fatwa is the belief that human life is sublime (expressed vividly in Surah Al Maeda i.e. Quranic Chapter entitled "the Feast": "Whoever saves one life, as if he saves all mankind".

بسم الله الرحمن الرحيم (ومن احياها فكانما احيا الناس جميعا) المائدة 32

Based on this and similar declarations, all Middle Eastern countries except Egypt passed laws that allow cadaveric transplantation and regulate live donations. Iran, Turkey, Saudi Arabia, Kuwait, Tunisia, Jordan, and Lebanon all have current active cadaveric programs and perform liver, heart, pancreas, and lung transplants [11, 12, 13]. The above Middle East Countries and others joined the Middle East Society for Organ Transplantation (**MESOT**).

Legal issues

In Iraq the legislation for organ (kidney) transplantation had been declared as follows [14]:

قانون عمليات زرع الاعضاء البشرية رقم (85) لسنة 1986

عنوان التشريع : قانون عمليات زرع الاعضاء البشرية رقم (85) لسنة 1986

التصنيف: قانون عراقي / رقم التشريع: 85 / سنة التشريع: 27 – 8 – 1986

مادة 1: يجوز اجراء عمليات زرع الاعضاء ل لمرضى بهدف تحقيق مصلحة علاجية راجحة لهم تقتضيها المحافظة على حياتهم وذلك من قبل الطبيب الجراح الاختصاصي في المركز الطبي المخول رسميا الذي يعمل فيه شريطة ان يكون هذا المركز معدا لاجراء عمليات زرع الاعضاء البشرية .

مادة 2: يتم الحصول على الاعضاء لاجل اجراء عمليات الزرع من:

أ. من يتبرع بها او يوصي بها حال حياته شريطة ان يكون كامل الاهلية عند التبرع او الايصاء وباقرار كتابي.

ب. المصاب بموت الدماغ وحسب الادلة العلمية الحديثة المعمول بها التي تصدر بتعليمات في حالة موافقة احد اقاربه الكامل الاهلية من الدرجة الاولى او الدرجة الثانية وموافقة لجنة مشكلة من ثلاثة اطباء اختصاصيين بضمنهم طبيب اختصاص بالامراض العصبية على ان لا يكون من بينهم الطبيب المعالج ولا الطبيب الاختصاصي المنفذ للعملية.

مادة 3: يمنع بيع وشراء الاعضاء باي وسيلة ويمنع الطبيب الاختصاصي من اجراء العملية عند العلم بذلك.

مادة 4: يعاقب بالحبس مدة لا تزيد على سنة واحدة وبغرامة لا تزيد على الف دينار او باحدى هاتين العقوبتين كل من يخالف احكام هذا القانون .

International status

There are several nearby Middle East countries with active KT programs from living and BD donors. The International Registry in Organ

donation and Transplantation (IRODaT) website declares with regular update the current status of living and deceased (BD) organ transplantation all over the world including MESOT countries as shown in **figure (1)** [15]**.**

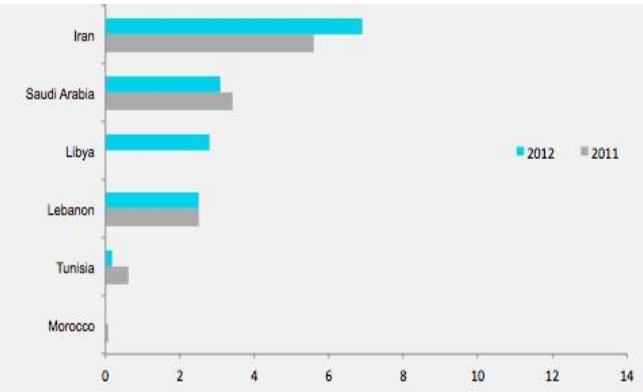

Fig. 1. Africa – Middle East Deceased Organ Donors per million population (PMP) 2012

In USA, the United Network for Organ Sharing (UNOS) which is a private, non-profit organization manages the nation's organ transplant system under contract with the federal government. UNOS developed an online database system, to collect, store, analyzes and publish all data related to the waiting list, organ matching, and transplants. Launched on October 25, 1999, this system contains data regarding every organ donation and transplant event occurring in the United States since 1986 [10].

Discussion

Iraq is one of the leading Middle East countries to start kidney transplantation (KT) program. The first living donor KT operation was carried out in 1973 by Professor Waleed Al Khial. The Medical City - Baghdad KT program started in 1985 by Professor Usama N. Rifat and colleagues. Since then KT was carried out in several governmental and private hospitals based on living related KT [16]. Although the legal and religious legislations for organ transplantation from BD donors are declared in our country in the 1980s, there is no yet a national BD organ donation program. This is related to several factors mainly the lack of awareness of BD organ donation merits from both health authorities and people in Iraq, lack of governmental economic funding for standardized ICU equipments and training the ICU staff to establish and maintain such program system, the series of social instability periods that Iraq passed through in the last decades due to wars and sanctions. In addition there is a lack of a standardized network system to collect and record all the relevant clinical data of kidney donation, transplantation and patients' follow up throughout the country.

Discussion of the national strategy to improve organ donation and transplantation in Iraq requires recruiting all the relevant authorities and professional staff who had experience of difficulties and limitations of organ transplantation to fix, re - write or modify the clinical and legal legislations concerning organ transplantation according to the current situation.

Regarding BD organ donation, it may be possible to think on the short term of cooperation of Iraqi Ministry of Health with certain regional / international authorities to bring cadeveric (BD) donor organs (Kidneys) to be used for transplantation and this requires legal and logistic infrastructure facilities to transport organs to the transplantation centre. This may go together with a national strategy on the long term to overcome lack of adequate public knowledge regarding organ transplantation from BD donors in Iraq by adequate and continuous public communication, education and organ donation campaigns for adults as well as educating high school and (medical and non medical) university students to promote a positive attitude towards organ donation among such young age group section of society. The latter can be implemented by teachers' education and highlighting the issue of organ failure and merits of life saving organ donation in the relevant teaching curriculum of students. The attitude of teachers [17] and high school students [18] toward organ donation was assessed in some countries as part of the above mentioned strategy. The same efforts should apply for upgrading our hospitals' ICUs and training medical and nursing staff working there

to maximize the clinical care for potential BD organ donors and achieve the best cooperation with the transplantation team including transplantation coordinators to avoid unnecessary delay of BD diagnosis and consequently improving the efficacy of organ donation program. Once people are aware of BD organ donation the next step to be implemented is the issuing of donor cards documenting the willingness of people wishing to donate organs when BD is established. Some Middle East countries published their experience in this respect [19].

The above steps have to go hand by hand with adequate support of transplantation surgeons by the ministry of health for updating their surgical experience and maintaining effective relevant research of patients' and allografts' survival [16] and postoperative complications as well as sharing the experience with the nearby MESOT recognized transplantation centers. The national plan to upgrade organ transplantation has to be carried out in a digital environment where all patients' clinical details are recorded in an intranet to facilitate retrieval and any necessary modification. This will clearly improve KT program from both living and BD donors and it will open the door for other organs transplantation in Iraq such as liver, cornea …etc to achieve the best results and saving more Iraqi patients suffering from end stage organ disease (ESOD). Finally it is of at most importance to thank all the Iraqi surgeons and other medical staff who worked hardly and continuously under all difficulties and limitations to initiate and maintain KT procedures which saved many Iraqi patients with ESOD

It is recommended to think of the regional and international experience of BD organ donation and open an intensive discussion to select the best plan which fits our local needs and our patient' safety.

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Outcome and Complications of Acute Intermittent Peritoneal Dialysis in Al-Kindi Teaching Hospital

Ali J Al-Saedi *FIBMS*

Dept. of Medicine, Al-Kindi College of Medicine, Baghdad University

Abstract

Background Acute peritoneal dialysis is commonly practiced in IRAQ.

Objective To evaluate and improve the management of peritoneal dialysis in Al-Kindi Teaching Hospital.

Methods Six hundred and sixty nine patients underwent peritoneal dialysis during the period from January 2011 to June 2012. The mean age of patients was 54±13.9 years. Among the patients, there were 168 cases of acute renal failure, and 501 cases of chronic renal failure requiring acute dialysis.

Results Acute peritoneal dialysis performed on patients admitted in dialysis unit of Al-Kindi Teaching Hospital was safe**,** a simple procedure, easily tolerated by the patient and requiring less expertise than hemodialysis and had complication rates nearly comparable to other established centers**.**
Complications occurred in 349 patients (52%). The most common complication was bleeding in the peritoneal cavity (30%) while dialysis solution leak was the second most common complication (25%). Dialysis episodes complicated by peritonitis was (2.5%). Death rate was 15%**.**

Conclusion Acute peritoneal dialysis was performed safely and effectively in Al-Kindi Teaching Hospital. Improvements are possible through closer supervision of new doctors and tighter nursing precaution.

Key Words Acute peritoneal dialysis, outcome.

Introduction

Peritoneal dialysis (PD) is a procedure that has gained widespread acceptance in the treatment of acute and chronic renal failure because of its simplicity and advantages compared with other modes of dialytic treatment such as hemodialysis (HD) [(1).]

This has led to the widespread use of peritoneal dialysis in the treatment of renal failure over the past four decades in many hospitals, both large and small [(2)].

It provides the nephrologists with nonvascular alternative for renal replacement therapy. It is an inexpensive modality in developing countries and does not require highly trained staff or a complex apparatus [(3)]. Systemic anticoagulation is not needed, and it can be easily initiated. It can be used as continuous or intermittent procedure and, due to slow fluid and solute removal, helps maintain hemodynamic stability especially in patients admitted to the intensive care unit. PD has been successfully used in acute kidney injury (AKI) involving patients with hemodynamic instability, those at risk of bleeding, and infants and children with AKI or circulatory failure [(4)].

The intention of the study is to evaluate the outcome and complications of acute intermittent peritoneal dialysis (IPD) in Al-Kindi Teaching Hospital, Baghdad, Iraq.

Methods

A cross sectional study was done from January 2011 to June 2012 on adult patients admitted to dialysis unit in Al-Kindi Teaching Hospital who had undergone PD. we designed a form that staff completed to evaluate the cause of renal failure, the indications for dialysis, the complications and outcome of PD. The technique of insertion of peritoneal dialysis is described below.

The patient was asked to empty his/her bladder before the procedure. An area below the umbilicus was prepared aseptically and a point at the middle third from the umbilicus to symphysis pubis was infiltrated with lignocaine. A small skin incision was made and the abdomen punctured vertically with firm pressure on the stylet catheter. The stylet was withdrawn slightly and the catheter introduced to either the iliac fossa posteriorly or to the pelvis.

The infusion tubing was then connected using meticulous aseptic technique. The dialysate bottles (commercially prepared) were suspended from a high stand and the fluid was infused into the peritoneum by gravity. We planned for 72 cycles of IPD in all patients. Each cycle was prescribed to be 1 hour in duration, with a drain time of 10 minutes, a dwell time of 30 minutes, and an outflow time of 20 minutes. Each exchange volume (2 L) contained 1.7% dextrose. In patients with fluid overload, dialysate containing a higher concentration of dextrose was used. Intraperitoneal gentamycin was used 12hourly as a prophylactic measure.

The patients were monitored for any variation in heart rate, blood pressure, or respiratory rate and for hemorrhage, catheter leak, catheter blockage, and infection. The serum creatinine, blood urea, sugar and serum electrolytes were determined daily while on PD. Peritoneal dialysate was sent for microscopic examination and culture only on suspicion of peritonitis. Peritonitis was diagnosed by the presence of turbidity of dialysate**,** abdominal pain / tenderness and fever.

The patients who refused to complete peritoneal dialysis for a cause other than development of complications (e.g. planning to consult other centers etc…) had been excluded in our study.

Statistical analysis

SPSS (statistical package for social science) version 16 software for windows was used. All data were entered and analyzed with appropriate statistical tests; Descriptive statistics were presented as (mean ± standard deviation), frequencies and percentages, Chi square was used for comparison of frequencies and percentage of different variables. In all statistical procedures and tests, level of significance was set at $P = 0.05$ was assumed.

Results

A total of 669 patients underwent IPD during the study period, including 382 men and 287 women. The age of the patients ranged from 18 years to 81 years with mean age 54 ± 13.9. Among the patients, there were 168 cases of acute renal failure (ARF) and 501 cases of chronic renal failure (CRF) requiring acute dialysis (Table 1). The underlying causes of acute and chronic renal failure are shown in (Table 2). Indications for acute dialysis were metabolic acidosis, uremic encephalopathy, fluid overload, hyperkalemia, pericarditis and uremic symptoms (Table 3).

Table 1. Demographic features of the studied patients

Feature	Mean ± SD	Range
Age (yr)	54.6 ± 13.9	18 - 81
Sex	Male Female	382 (57.1%) 287 (42.9%)
Renal failure	Acute Chronic	168 (25.11%) 501 (74.89%)

Table 2. Causes of renal failure of patients

Causes		No.	%
Acute renal failure	Acute tubular necrosis	80	11.96
	Rapidly progressive GN	35	5.23
	Acute interstitial nephritis	32	4.78
	Obstructive uropathy	11	1.64
	Hepato-renal syndrome	10	1.49
	Total	168	25.11
Chronic renal failure	Diabetic nephropathy	170	25.41
	Chronic GN	102	15.25
	Hypertension	94	14.05
	Chronic Pyelonephrits	72	10.76
	Obstructive uropathy	63	9.42
	Total	501	74.89
Total		669	100

Table 3. Indications of acute dialysis

Indication	No. of patients	%
Metabolic acidosis	214	31.98
Encephalopathy	169	25.26
Fluid overload	151	22.57
Hyperkalemia	82	12.26
Pericarditis	29	4.35
Uremic symptoms	24	3.58
Total	669	100

Efficiencies of PD for the purpose of this study were estimated using the differences in the pre and post blood urea level and clinical improvement. The mean urea lowering was 95±82 mg/dL. Serum biochemistry of patients prior to initiation of peritoneal dialysis was shown in (Table 4). Complications of PD were divided into mechanical, metabolic and infection (Table 5). The number of patients that developed complications were 349 carried a rate of (52%).

Table 4. Serum biochemistry of patients prior to initiation of peritoneal dialysis

Biochemistry	Range	Mean ± SD
Blood urea mg/dL	95 - 270	238 ± 26.7
Creatinine mg\dL	2.1 - 10.5	6.3 ± 0.65
Potassium mEq\L	3.2 - 5.8	4.7 ± 2.1
Sodium mEq\L	112 - 152	128.3 ± 6.3
Chloride mEq\L	90 - 104	95.2 ± 4.1
Total calcium mg\dL	7 - 10	7.9 ± 0.45
Phosphate mg\dL	3.3 - 6.1	5.36 ± 1.87
Albumin g\L	33 – 50	39.5 ± 7.1

Table 5. Complications of acute intermittent peritoneal dialysis

Complications*		Number of episodes	Percent
Mechanical	Bleeding	201	30
	Peritoneal solution leak	167	25
	Abdominal pain	147	22
	Bowel perforation	1	0.15
Metabolic	Hyperkalemia	132	20
	Hyperglycemia	33	5
Infection	Peritonitis	17	2.5

* The patient may develop more than one complication at the same time

Bleeding from peritoneal cavity occurred in 201 episodes of dialysis, 143 of which were mild, 40 were moderate and 11 were severe bleeding required blood transfusion.

Dialysis solution leak occurred in 167 of the cases, 131 of them necessitated reinsertion of the catheter. One hundred forty seven patients complained of catheter-related pain. After an explanation and reassurance, 110 of them tolerated the pain. Thirty seven patients treated with analgesia for pain relief. Bowel perforation occurred in one patient who improved on conservative treatment and referred for HD.

Peritonitis occurred in 17 patients and all presented with turbid effluent fluid. Culture was positive in 12. Nine yielded mixed growth of gram negative bacilli, two grew *staphylococcus aureus*and the one grew *pseudomonas aerogenes.* Treatment in 9 cases was intravenous cefotaxime. In two other cases intravenous vancomycin was used, last patient was treated with piperacillin.

PD catheters were removed in all cases to control the unsettled infection. Treatment was successful in all except three patients who died of overwhelming septicemia. Hypokalemia occurred in 132 patients and hyperglycemia in 33 patients. They were managed with potassium and insulin respectively. There were one hundred deaths during this study period and the causes of death are shown in Table 6.

Table 6. Causes of death in patients on intermittent peritoneal dialysis

Causes of death	Acute renal failure		Chronic renal failure		Total no.	P value
	No.	%	No.	%		
Septicemia	33	49.25	6	18.18	39	0.005
Brain stem stroke	20	29.85	16	48.48	36	0.11
Acute leukemia	3	4.48	-	-	3	0.54
Myocardial Infarction	8	11.94	7	21.21	15	0.36
Pericardial temponade	-	-	4	12.12	4	0.55
Hepato-renal syndrome	3	4.48	-	-	3	0.54
Total	67	100	33	100	100	0.001

Sixty seven of the patients who died had acute renal failure while the remaining thirty three patients had chronic renal failure. Deaths were due to the underlying disease and not to uremia as plasma biochemistry was well controlled in PD. Only four of the deaths can be attributed directly to uremia. Those patients developed pericardial tamponade soon after the initiation of PD. They presented with symptoms and signs of uremia. Their blood pressure was initially

high, jugular venous pressure was elevated. Heart sounds were clearly audible and there was no pericardial rub. While they were on PD, they suddenly became breathless and hypotensive. An echocardiogram was done for two of them which revealed massive pericardial effusion with features of pericardial tamponade. Their blood pressure returned to its original baseline following pericardiocentesis. Unfortunately, the improvement in blood pressure was not sustained. They became hypotensive again and this time they did not respond to resuscitation. Uremic pericarditis with pericardial tamponade was presumed to be the cause of their death as the pericardial effusion aspirated was blood-stained and they were still clinically uremic with the blood urea prior to their death being high. The relatives of other two patients refused any further intervention and eventually they died.

Discussion

Peritoneal dialysis is performed frequently in Al-Kindi Teaching Hospital. This frequency gives a fair chance for doctors and nurses to be skillful in the technique and nursing care of the dialysis procedure.

Peritoneal dialysis is a simple procedure that can be started easily and without delay. The PD treatment modality is invaluable in patients with ARF, in whom short-term dialysis support can be life-saving and can affect a complete cure. Similarly, in patients with CRF, in whom various aggravating factors have caused acute exacerbation of their illness, short-term dialysis support can help both to reverse the acute component and to treat the precipitating factors. With restoration of renal function to baseline level, patients may remain independent of dialysis for several months or years.

The mortality rate in patients on PD has been reported to vary between 5% - 12 % [5]. Mortality in our study was 15% which is nearly comparable to a study in Koirala Institute of Health Sciences in Eastern Nepal at 2003 (death rate was 12.5%). Sixty seven out of one hundred sixty eight (40%) patients with acute renal failure died compared to thirty three out of five hundred one patients (6.5%) with chronic renal failure (statistically significant). It has been shown in most series that patients with acute renal failure have higher mortality rates because of concomitant medical problems. This was well illustrated in our study where deaths were attributed to the severe underlying disease rather than to uremia. Only four deaths could be directly attributed to uremia. Those patients had pericardial tamponade due to uraemic pericarditis soon after initiation of PD.

Vaamonde and Valk [5] reported 30-32% of dialysis was complicated by bleeding most of which were minor. In our study 30% of patients had bleeding (majority was mild) which is nearly comparable with bleeding rate (20%) in a study that done in Hospital University Science Malaysia Zainaland Loo 1992. Uremic patients invariably have abnormalities of platelet function characterized by a prolonged bleeding time, abnormal platelet aggregation, abnormal platelet adhesion test and decreased release of platelet factor 3 [6,7]. The platelet count is generally normal and alteration in the concentration of circulating clotting factors, when present, is not consistent and does not contribute to a bleeding tendency. The result that correlates best with the occurrence of clinical bleeding is the abnormality of bleeding time [6]. Even though detailed platelet function tests have not been carried out, the presence of prolonged bleeding time, normal platelet counts and the appropriate clinical setting have allowed us to conclude that bleeding in our patients was due to uremia. We continued with PD despite bleeding from the peritoneal cavity. The bleeding ceased while the patients were on PD and this further supported our initial impression that the bleeding was uremic in nature. Other therapeutic modalities that have been shown to correct bleeding time of uremia are infusion of cryoprecipitate [8] and injection of l-deamino-8D-arginine vasopressin [9] and oral or parenteral administration of a conjugated estrogen preparation [10] The above measures were not used in our patients (not available) apart from

cryoprecipitate. Those patients who developed severe bleeding had different hematological disorders.

Peritonitis was a potentially serious complication. It occurred in 17 patients, giving a rate of 2.5%. This rate is low as compared with 17.2% recorded in the General Hospital Kuala Lumpur [11] and 15% in Hospital University Science Malaysia Zainal and Loo, 1992

However, rate is comparable with 0.1% to 2% which have been quoted in the literature [12,13]. The rate of peritonitis can be lowered by meticulous attention to aseptic technique during catheter insertion, followed by careful nursing care and the use of intra peritoneal prophylactic antibiotic.

The isolation of gram-negative organisms from the effluent fluid in nine out of twelve cases with positive culture was surprising. This may imply the presence of unsuspected intra-abdominal pathology.

Bowel perforation occurred in one patient (0.1%) which is low as compared with (2.5%) in Hospital University Science Malaysia.

The patient recovered well on antibiotics and was converted to hemodialysis. It is important to note that while treatment of bowel perforation may be conservative in some cases, most require laparotomy [13,14] and this complication may be fatal[15].

In conclusion, acute (stab) peritoneal dialysis was performed safely and effectively in Al-KindiTeaching Hospital, complication rates were nearly comparable to other studies, bleeding that occurred during PD was due to effect of uremia on platelet function, the rate of peritonitis can be lowered by using highly aseptic technique during catheter insertion and careful nursing care, use of intra peritoneal prophylactic antibiotic may have a role in decreasing the rate of peritonitis, and acute renal failure is associated with higher mortality rate and is related to underling disease and co morbidity, rather than PD procedure itself.

Further study is recommended to assess the quantitative effect of PD in removal of excessive body fluid, to correlate between the delay in initiation of PD and the rate of complications which definitely increase due to delayed referral, continuous type of PD is recommended in our country as a type of renal replacement therapy for both ARF and CRF especially for those who have contraindications to HD.

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E-mail: dralijasim@yahoo.com
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Postprandial Triglyceride and Testosterone in Women with Cardiovascular Diseases

Shaymaa Z. Al-Saedi[1] *MSc*, **Ghassan A. Al-Shamma**[1]*PhD*, **Hashim M. Hashim**[2]*MRCP*

[1]Dept. of Chemistry and Biochemistry, [2]Dept. of Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background High androgen levels may increase cardiovascular disease (CVD) risk in women through adverse effects on lipids, blood pressure, and glucose metabolism. Lipid abnormalitiesare often found in women with CVD.

Objective To study the relationship between postprandial triglycerides (TG) as a risk factor for cardiac disease and the androgenic activity in postmenopausal women with CVD.

Methods Postprandial lipid profile and sex hormone levels were measured in 30 patients with CVD and 25 postmenopausal women age and body mass Index (BMI) matched served as control group.Testosterone and sex hormone binding globulin (SHBG), Estradiol (E2), follicular stimulating hormone (FSH) and luteinizing hormone (LH) were estimated.

Results Postprandial TG, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and atherogenic index (AI) were different between the two groups ($P \leq 0.001$). The SHBG and Free Androgenic Index (FAI) were significantly higher in the CVD postmenopausal women ($P \leq 0.001$) while no differences in testosterone, LH, FSH, and E2 levels noticed between CVD patients and the control group. Serum testosterone levels correlated positively with the postprandial TG and the atherogenic index, and negatively with HDL-C in the CVD patients.

Conclusion Elevation in androgenic activity could be a cause of higher elevation in postprandial serum TG which may increase the risk of CVD in women.

Key Words postmenopausal women, cardiovascular disease, postprandial lipid profile, testosterone, sex hormone binding globulin, free androgenic index

Introduction

Cardiovascular disease (CVD) is the leading cause of death in women [1,2]. Postprandial triglyceride (TG) concentrations areoften elevated throughout the day,a point which makes postprandial TG concentration a better predictor of cardiovascular events than fasting triglycerides [3-5]. The adverse effect of postprandial TG is thought to be mediated by the pro-atherogenic lipolysis products of nascent triglyceride-rich lipoproteins, which may worsen vascular function [3-5].

In subsequent analysis of a larger number of men and women, non-fasting TG was not associated with coronary death in men but showed a 5-fold risk of death from coronary heart disease in women when its concentration was 3.5 mmol/L,or more, as compared to those with a level of less than 1.5 mmol/L, even after adjustment for traditional coronary risk factors [6].

The aim of the present study was to emphasize the association of postprandial rise in serum TG with changes in sex hormones in women with CVD and their role in increasing the risk of CVD.

Methods

Thirty patients with CVD aged between 48-63 years (55±8.9, mean±SD) were recruited from Al-Imamain Al-Kadhimyian Medical City during the period from January to April2012. Another 25 apparently healthypostmenopausal women were involved as a control group with matching age and body mass index (BMI) to the patient group (53.3±6.7years, mean±SD). None of them had a history of thyroid disease, polycystic ovary syndrome (PCOS), diabetes mellitus, renal impairment, or any other severe illness or infection, and not taking any drug (including hormone replacement and any estrogenic, anti hypertensive or lipid lowering medication) or had any operation in the ovary. Ten mls of blood were collected in a plain tube in postprandial state (2-3 hours after breakfast). The serum was obtained after centrifugation at 3200 rpm for 10 min. and divided into small aliquots.

a- Immediate measurements of serum glucose, lipid profile, were done using the enzymatic colorimetric methods.

b- The rest was stored at - 20^{o}C until assayed for hormones analysis (luteinizing hormone (LH), follicular stimulating hormone (FSH), and estradiol (E2)) by mini VIDAS Kit (Biomerieux, France), while testosterone and sex hormone binding globulin (SHBG) wereestimated by manual Eliza kit.

c- Free Androgen Index (FAI) was calculated by using the formula total testosterone (mmol/ L) / SHBG (mmol/L).

d- Body mass index (BMI) was calculated by weight (Kg)/sq height (m)

e- The atherogenic index = LDL.C / HDL.C

Results

The results show highly significant elevations in the postprandial TG, TC LDL-C, and atherogenic index, with a highly significant reduction in HDL-C (p-value = 0.0001) in the postmenopausal women with CVD as shown in table 1 when compared to their healthy controls.

Table 1. Demographic features of postmenopausal women with cardiovascular disease and the control group

Parameters	Women with CVD N = 30	Control group N = 25
Age (year)	55±8.9	53.3±6.7
BMI (kg/m^2)	30.9±2.4	29±3.1
Triglyceride	3.82±0.85*	2.45±0.34
Total cholesterol	6.41±0.48*	5.17±0.11
HDL-C	0.65±0.26*	0.93±0.06
LDL-C	3.82±0.44*	3.13± 0.14
Atherogenic index	4.93±1.48*	3.37±0.68

* $P \leq 0.0001$, CVD = cardiovascular disease BMI = body mass index, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol.

As shown in table 2 there was no significant differences in testosterone, LH, FSH and E2 levels in postmenopausal women with CVD and postmenopausal women without CVD (p = 0.62, 0.78, 0.27 and 0.057 respectively), with a highly significant reduction in SHBG levels (P = 0.0001), however, the free androgenic index was significantly higher inthe postmenopausal women with CVD than their controls (menopausal women without CVD, P = 0.001).

Table 2. Sex hormones & Free Androgenic Indexin thepostmenopausal women with CVD and control group

Parameters	Women with CVD N = 30	Control group N = 25
LH (mIU/ml)	41.44±4.08	38.46±5.42
FSH (mIU/ml)	45.39±3.19	41.74±6.31
E2(pg/ml)	59.73±15.99	50.32±4.11
Testosterone (nmol/l)	2.92±0.54	2.43±0.27
SHBG (nmol/l)	61.23±10.54*	71.61±5.41
FAI	5.66±1.67*	3.38±0.35

*P ≤ 0.0001, CVD = cardiovascular disease, LH = luteinizing hormone, FSH = follicular stimulating hormone,E2 = estradiol, SHBG =sex hormone binding globulin, FAI = free androgenic index.

Discussion

The significant increase in postprandialserum TG, total and LDL cholesterols and atherogenic index with a significant reduction in HDL-C seen in this study contributes to the irregular lipid metabolism in postmenopausal women with CVD when compared to the healthy postmenopausal women.

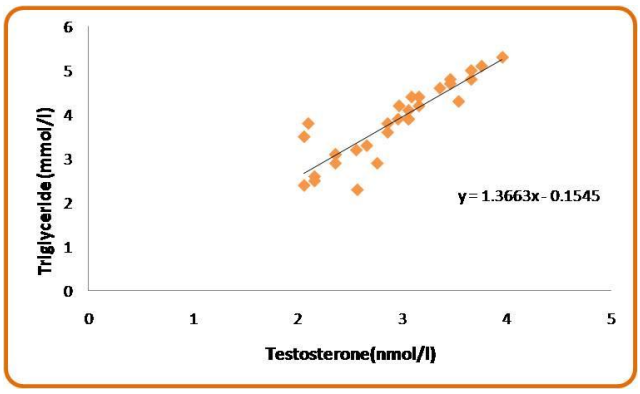

Fig. 1. The correlation between testosterone level and postprandial triglyceride in the postmenopausal women with cardiovascular disease

Studies had related these abnormalities in lipid profile to insulin resistance, which may cause elevation in LH and testosterone and reduction in FSH, E2 and SHBG [7,8]. The LH stimulates theca cells resulting in production of testosterone and androstenedione, whereas the FSH stimulates aromatase in the granulose cells, resulting in aromatization of androgens to estrogens [9-11]. The reduction in SHBG is related to hyper-insulinemia due to the ability of insulin to inhibit hepatic SHBG synthesis [12]. From the calculated value of FAI, whichdetects free testosterone, it could be said that the loss in circulating SHBG leads to greater bioactivity of circulating testosterone.

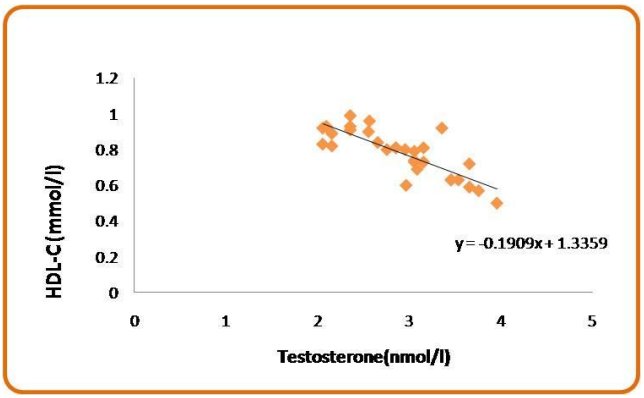

Fig. 2. The correlation between serum testosterone level and postprandial high density lipoprotein cholesterol in the postmenopausal women with cardiovascular disease

Measurement of postprandial serum lipids has been recommended as a better marker than fasting serum lipids for many diseases including the CVD, as it would catch the peak of serum TG during the 2-4 hours after meals which is believed to play an important role in the preparation for the process of atherosclerosis [13,14].

Previous studies showed that SHBG may mediate its positive effect on the lipid profile by regulating bioavailable androgen levels. SHBG binds testosterone with high affinity and regulating its free concentration [15,16].

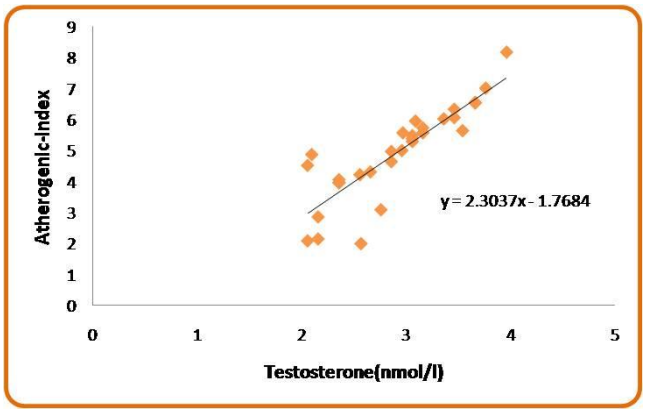

Fig. 3. The correlation between serum testosterone level and atherogenic index in the postmenopausal women with cardiovascular disease

Reports on the relationship between SHBG and CVD were controversial: Low SHBG levels, sometimes wereconsidered androgenic marker in women, demonstrating a positive correlation between SHBG and HDL-C and a negative correlation with more atherogenic lipid profile total and LDL cholesterols [17-18] while another study failed to find such an association [19].

In this study the significant correlations between testosterone and various postprandial serum lipids in the postmenopausal women with CVD (Fig.1-3) emphasize the association between the two parameters and may lead to the suggestion that the increase in androgenic activity (or free testosterone) may increase the elevation in postprandial TG which is believed to be a cause of increased risk of CVD in women [13,14].

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Correspondence to Dr. Ghassan A. Al-Shamma
E- mail: ghassan.1971@yahoo.com.
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Iliac Crest Bone Graft in Maxillofacial Bony Defects

Ayad A. Hasan *FICMS*, **Ammar Y. Khudhir** *FICMS*

Section of Maxillofacial Surgery, Dept. of Surgery, Al-Imamian Al-Kadhymain Medical City, Baghdad, Iraq.

Abstract

Background The reconstruction of facial deformity has been of paramount clinical concern for many years and one of the most difficult and challenging tasks facing the maxillofacial surgeon. The ultimate goal in the treatment is the relief of suffering, restoration of function of jaw, restoration of speech, regain of the normal looking contour and improvement in the quality of life.

Objective To obtain more knowledge on autogenous on lay bone graft behavior in different facial defects and to evaluate the lateral and medial surgical approaches to the iliac crest.

Methods A prospective study was conducted during the period from January 2009 to January 2012 on 20 patients with facial defects, in the orbit, zygoma, maxilla and mandible. The causes of defects were trauma, odontogenic tumors and alveolar cleft. Types of bone graft used were either block cortico-cancellous or chips cancellous bone, the block was either monocortical or bicortical bone graft.

Results Complications associated with donor site harvesting procedure included pain 5% and gait disturbance 5%. Failure of bone graft was observed in 3 patients (15%) while the rest, 17 patients, (85%) ended with functional and esthetic successful graft. Causes of failure were due to sequestration (5%), inflammation due to osteomesh (5%) and soft tissue breakdown (5%).

Conclusion Iliac crest graft has evolved as a safe, well accepted procedure, with relatively low morbidity that can be used for a wide variety of maxillofacial procedures.

Keywords Iliac crest, bone graft, facial defects

Introduction

The reconstruction of facial deformity has been of paramount clinical concern for many years and one of the most difficult and challenging tasks facing the maxillofacial surgeon. Bone graft represents one of the earliest devised reconstruction approaches to the musculoskeletal system. In addition, autogenous bone is available in many forms and unlimited quantity [1,2].

The ultimate goal in the treatment is the relief of suffering, restoration of function of jaw, restoration of speech, reprintof the most normal looking contour, and improvement in the quality of life [3-5].

In osseous restoration of full bone thickness for the face, many factors render the usual bone grafting procedures difficult and their prognosis uncertain including contour, morphology, nature of the muscular attachments and the effects of continuing trauma associated with mastication, phonation, swallowing and other mandibular movements [6,7].

The use of cortical and cancellous bone graft is common in many craniofacial deformities, because of its accessibility and the quantity of bone available. The ilium has become a

favorable donor site for cortico-cancellous and marrow grafting material [8-10]. Cortical bone, also known as compact bone, is one of two main types of osseous tissue. Cortical bone is dense, and forms the surface of bones. It is solid in appearance, and constitutes 80% of total bone mass. Compact bone is composed of many cylinder shaped units called osteons, or Haversian Systems, and transverse channels between them called Volkmann's Canal.

Cancellous bone, synonymous with trabecular bone or spongy bone, is one of two types of osseous tissue that forms bones. Compared to compact bone, which is the other type of osseous tissue, it has a higher surface area but is less dense, softer, weaker, and less stiff. It typically occurs at the ends of long bones, proximal to joints and within the interior of vertebrae. Cancellous bone is highly vascular and frequently contains red bone marrow where hematopoiesis, the production of blood cells, occurs. The primary anatomical and functional unit of cancellous bone is the trabecula. The bone usually forms initially as cancellous bone and then forms the compact bone [11].

The objectives of this study was to obtain more knowledge on autogenous on lay bone graft behavior in different facial defects and to evaluate the lateral and medial surgical approaches to the iliac crest.

Methods

Data were obtained in this study by reviewing prospectively the results of 20 patients with facial bone defects, 13 patients were males, and 7 were females with an age ranged from 12 to 59 years. All of the patients were subjected to autogenous bone graft taken from the anterior iliac crest to reconstitute facial defects. Those patients were admitted to the Department of Maxillofacial Surgery in Al-Imamain Al-Kadhymain Medical City during the period from January 2009 to January 2012.

The causes of defects were trauma, odontogenic tumors and alveolar clefts. The bone graft was used either immediately or after a while. The types of bone grafts used were either block corticocancellous or chips cancellous bone. The blocks were either monocortical or bicortical bone graft. The bone grafts were fixed by wires, bone plates, and screw by the use of osteomesh in case of chips bone graft; and for the mandible, intermaxillary fixation was used for immobilization for six weeks duration. Follow up period ranged from 3 months to 2 years after operation.

The indications for the orbital defects were cosmetic, and elimination of diplopia due to blow-out fracture. For the zygoma, the indication was cosmetic. For the maxillary and mandibular defects, the indications were closure of alveolar clefts, preparing a good bone quality for the placement of dental implant, severe trauma with loss of major part from the mandible and correction of the contour of the mandible after excision of a tumor. Table (1) shows the location of the defects.

Table 1. The different locations of the bone defects

Location	No.	%
Orbit	4	20
Zygoma	4	15
Maxilla	3	15
Mandible	9	45
Total	20	100

In regard to the type of treatment, the patients were treated as follows: for orbital defects, 4 patients with defective floor, 3 on the right side and one on the left side, 2 due to missile injury and 2 due to Rather infra-orbital approach was used within skin creases; and all of the bone grafts used were corticocancellous monocortical bone graft. Size of the defect was about 3 cm. The extensions of defect for grafting are shown in (Table 2).

Four patients had zygomatic bone defects, 3 on the left side, one on the right side. The treatmentwas done by extra-oral approach, infra-orbital approach, and cortico-cancellous monocortical bone graft was used. Size of the defect ranged from 3-5 cm.

Table 2. The extension of the defects needs to be grafted

Area	Size of the defects
Orbit	3 cm
Zygoma	3cm-5cm
Maxilla	2cm-4cm
Mandible	4cm-10cm

For the maxilla, there were totally 3 cases, 2 cases with alveolar cleft and 1 case with trauma to the alveolar ridge due to shell injury.Intra-oral approach was used and we inserted a monocortical corticocancellous bone graft to form an alveolar ridge in order to make an implant for the upper jaw. Size of the defect ranged from 2-4 cm.

For the mandibular bone defects, there were a total number of 9 patients operated for reconstruction of mandible, caused by RTA, shell injuries and ameloblastoma. The types of bone graft used were either cortico-cancellous bone graft, monocortical or bicortical, or chips of cancellous bone graft inserted in osteomesh tray at the defect site. The approach was always conducted through submandibular incision, and immobilization of the mandible was done by arch bar and interaxillary fixation for at least 4 weeks (between 4-6 weeks). Size of the defects ranged from 4cm –10 cm.

The patients were operated for bone graft taken from anterior iliac crest by two approaches; in 13 patients, we used medial approach, and in 7patients, we used lateral approach. A trapdoor osteotomy technique was usedfor all of the patients by retracting the skin by the assistant and incision was extended through the skin and periosteum to the crest of the ilium so that the incision lies lateral to and below the crest. The periosteum was reflected and raised with periosteal elevator. By vertical sectioning of the portion of the crest between the vertical cuts, a section of cortical table with its underlying cancellousbone was done. When cancellous bone alone was needed, an osteotomy was made over the central portion of the iliac crest and a wedge of cancellous bone is resected. The outer and inner tables of the ilium were then fractured toward each other with heavy forceps in order to eliminate the resulting dead space between them. This technique did not disturb the continuity of the crest and left no visible deformity. The amount of bone volume was measured visually. The drain was inserted and removed later when the amount of blood was less than 20 cc, removed from the donor site.

Results

The age of the patients (Table 3) with bone graft for reconstruction of facial defects ranged between 12 and 59 years. The mean age of the patients was 33.6 years. The male percentage operated on were 65% (13 males), and the percentage of females were 35% (7 females).

Table 3. Age of the patients

Age range (yrs)	No.	%
10 – 20	2	10
20 – 30	3	15
30 – 40	12	60
40 – 50	2	10
50 – 60	1	5
Total	20	100

The anatomical region distribution for facial defects that required bone graft were as follows: for orbit defects,20%, zygomatic defects, as shown in Fig.8 and 9, 20%, maxillary defects (Fig.1,2 and 6) 15% and mandibular defects (Fig. 8)45%.

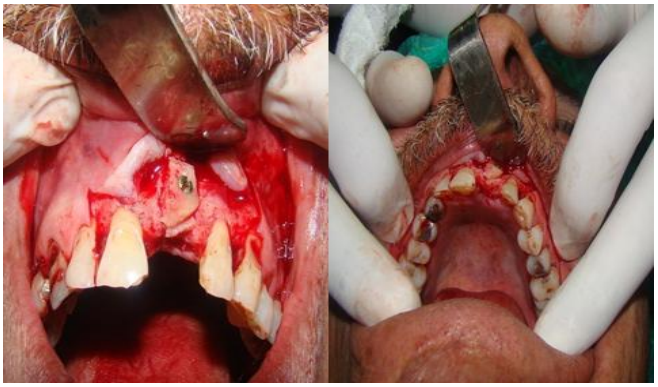

Fig. 1. Bone graft inserted to the alveolar bone and fixed by screw only to prepare a bed for dental implant. The cause of this defect was due to shell that caused avulsion of the left central incisor tooth and a part of the alveolar bone.

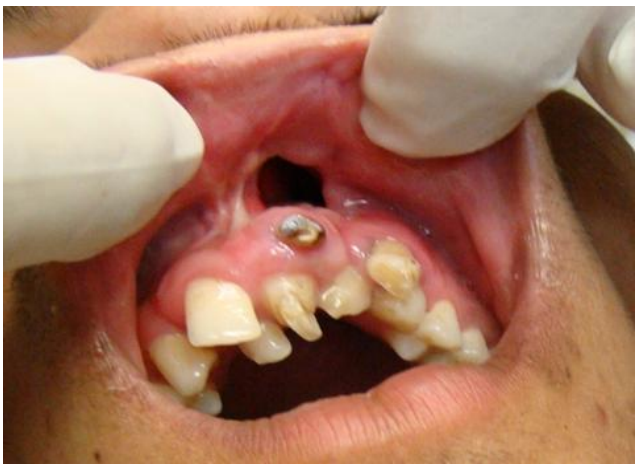

Fig. 2. Alveolar cleft.

The etiological factors of bone defects were 65% due to trauma, and from these 65% traumatic causes, 35% were due to RTA, 10% were bullet injuries and 20% were shell injuries. Alveolar clefts causes occur in 10% and odontogenic tumors of the mandible in 25% of cases (Table4). The immediate method of reconstruction was used in 5 patients (25%), mainly used in treatment of odontogenic tumor lesions. The delayed reconstruction due to trauma and other causes used in 15 patients (75%).

The method of immobilization of the mandible to the maxilla was done by intermaxillary fixation to the patients with mandibular defects in 9 patients (45%), while the zygomatic defects, orbital defects and maxillary defects did not need intermaxillary fixation.

Table 4. Causes of the bone defects

Causes of the defects	No.	%
Road Traffic Accidents	7	35
Bullet Injury	2	10
Shell Injury	4	20
Odontogenic Tumors	5	25
Alveolar cleft	2	10
Total	20	100

Complications associated with donor site harvesting procedure include pain in 1 patient (5%) persist for about 4 weeks and treated by analgesics and anti-inflammatory medications. Gait disturbance occur in 1 patient (5%) treated by physiotherapy, and improved. No complication occurs in 18 patients (90%).
Failure of bone graft observed in 3 patients (15%) while the rest 17 patients 85% ended with functional and esthetic successful graft (Table 5).

Table 5. Classification of Failure cases

Failure cases (Total no= 3) (15%)	Number of patients	%
causes	1 sequestration 1 inflammation from osteomesh 1 soft tissue breakdown	5 5 5
Types of bone graft	1 cancellous bone 2 corticocancellous	5 10
Defect involvement	2 bone and soft tissue 1 bone loss alone	10 5
Etiological factors	1 ameloblastoma 2 shell injury	5 10
Anatomical distribution of the defect	1 alveolar bone 1 mandible 1 zygoma	5 5 5

Discussion

The bone formation within the graft was more abundant when an iliac graft was used, probably because of better survival of osteogenic cells and more complete vascularization within the graft [11-13].

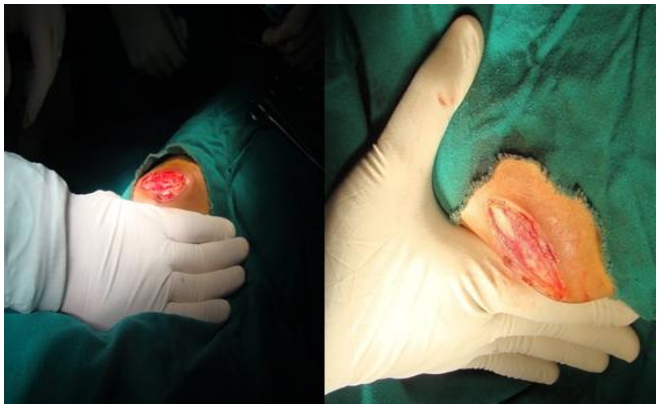

Fig. 3. Incision of the skin of the donor site

In reviewing the traumatic cases operated for bone graft, we found that the most etiological factors for the bony defects were high speed vehicles. This result is not similar to the study of Poirit (1953) which showed that 75% of maxillofacial injuries of the 2nd war were due to the fragmentation missiles, which in our study were the 2nd most common cause of facial defects [14]. Olsen and colleagues 1982 also reported that the most common cause of the defect was due to vehicle accidents [15].

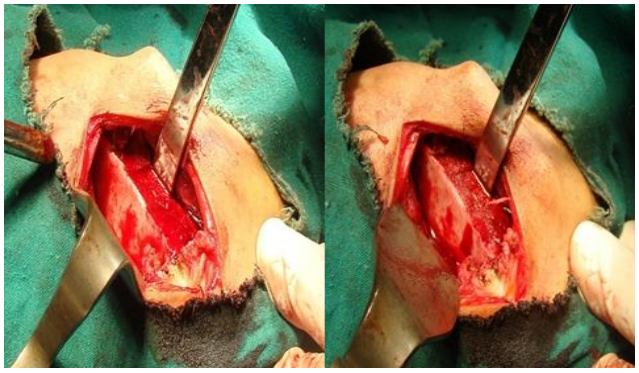

Fig. 4. Bone graft was taken as one block

Most surgeons who carried out bone grafting for mandible as immediate reconstitution to restore the defect caused by tumor surgery and not by a traumatic injuries. The chance for successful bone graft is higher if there is an adequate time elapsed between the time of injury and the time of surgical procedure in cases of traumatic injuries; this is due to risk of infection and sequestration of fragmented bone pieces as consequence to trauma [16]. So, the policy of our treatment in cases of traumatic injuries was to wait until all signs of infection disappeared, but certain steps of preparation were done to make the recipient site ready for grafting. While for odontogenic tumors we treated them immediately after removal of the tumor from the mandible.

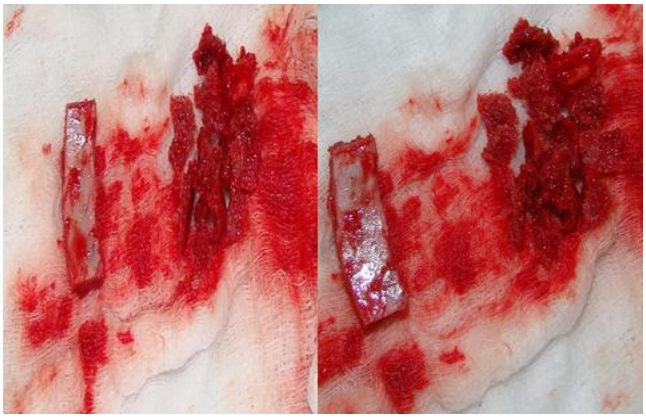

Fig. 5. Cortical and cancellous bone graft taken from the anterior iliac crest

Komisar (1985) concluded that delayed reconstruction was superior due to absence of infection and the immediate reconstruction of mandible has more chance to fail, while the delayed repair of traumatic injuries has better results [17]. Lundgren (1999), favor the use of a delayed approach by free autogenous bone graft [18].

Immediate mandibular reconstruction can be performed safely and expeditiously in nearly all healthy patients undergoing segmental mandibulectomy [19].

In our study, the cases were treated either by corticocancellous block bone graft or cancellous chips bone graft depending on the site and the length of the defect and the bed of recipient site (Fig.3-5). When the defect occurs in the mandible and extended to involve the body of

the mandible, Dacron-urethane mesh was used to carry the particular bone graft. In the case of defects in the floor of the orbit, zygoma, alveolar area, maxilla and the body of the mandible, a block of corticocancellous bone graft was used to repair the defect. There was no difference between the two types of bone graft with reference to their failure, but surgeons must adhere strictly to adequate preoperative preparation and skillful technique including generous graft contact and rigid stabilization.The graft bed must be vascular and free from infection. These should be observed in any procedure of bone grafting [20,21].

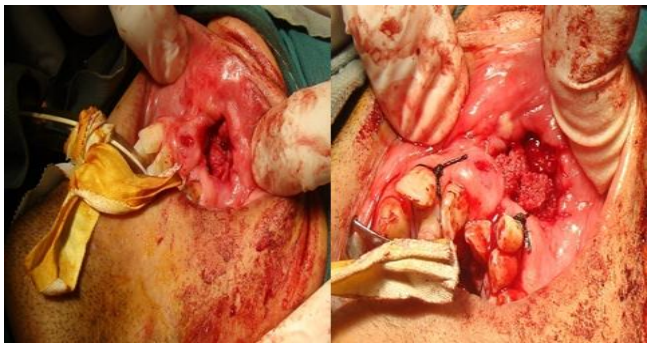

Fig. 6. Recipient site

Upon reviewing of our cases and being compared with those of other surgeons, we agree with Schwartz (1984) who stated that There is no all-purpose type of bone graft [22], and proper technical procedure with healthy non-scarred bed and rigid fixation will make the bone graft appear better than cancellous chip of bone because it is more rigid and helps in fixation. The mandibular osteomesh may stimulate a low-grade inflammation with seroma formation. The use of cancellous bone needs more care about fixation and stabilization which are not provided by its tray alone.

In all our cases treated by bone grafts, transosseous wires, screws and miniplates introduced to hold the corticocancellous bone graft to the bone of recipient site as well as the mandibular tray used to carry the spongy bone graft of the mandible. For immobilization mainly for mandibular bone graft, we used IMF. We think thatcases of rigid internal fixation are very good and donot need IMF for the mandibular reconstruction. The survival and success of the bone graft dependson the rapidity of the revascularization and the 1st phase of bone healing process can only take place if the capillary growth into the transplant finds absolute stable condition; therefore, secure internal fixation of the graft is mandatory [23].

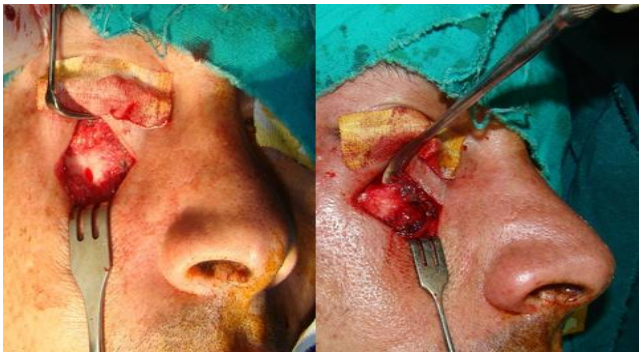

Fig. 7. Zygomatic bone defects (contour deficient due to old trauma)

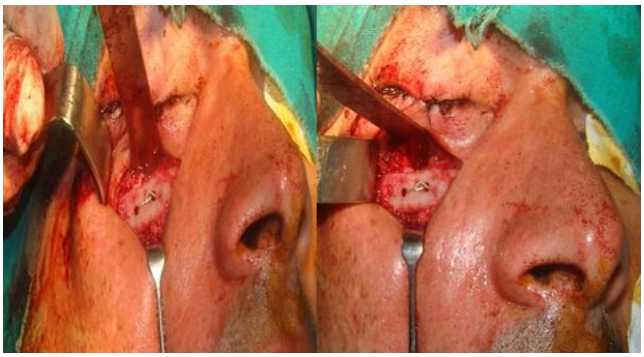

Fig.8. Preparation and fixation of the iliac crest bone graft to the zygomatic defect by stainless steel wire

Some surgeons were more conservative[24], in that they maintain their internal rigid fixation with reconstruction plates, lay screws, as well as miniplates for 3-4 months and then remove it to expose the bone transplant to functional load. Comparable with our cases where osteomesh tray was used, other surgeons showedlow modulus of elasticity lesser than that of cortical bone. Permitting the functional stress which may be an important factor in extensive graft resorption when titanium tray or bone plates are used, the extensive rigid fixation with high

modulus of elasticity was found to prevent the stress from transmitting to the bone graft [24].

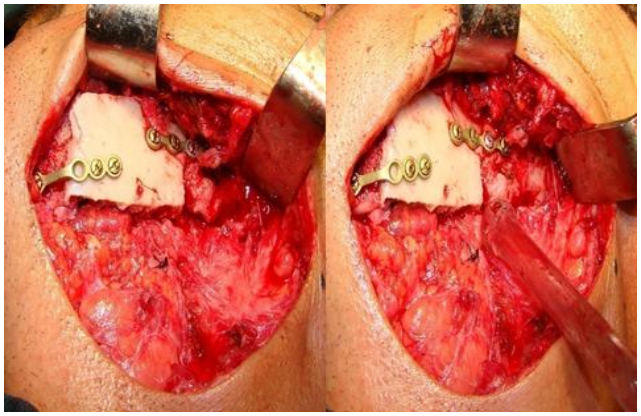

Fig. 9. Iliac crest bone graft inserted and fixed to the mandible immediately after segmental resection of the mandible due to ameloblastoma

The use of transosseous wire in compact bone graft gives a secure fixation when applied at each end by two holes for the recipient site and the bone graft. The wires passed through the holes and twisted tightly as conventional type and figure of eight wire but it was not regarded as a type of rigid fixation.

Ralf et al. suggests that miniplates fixation can be regarded as the method of choice for fixation of bone graft [25].

The recipient site needs not to put drain because the postoperative hematoma and seroma can be prohibited by arresting of all the bleeders, closure of the wound in layers and dressing with a pressure pack.

The most significant cause of early donor site morbidity was local pain, lasting up to 4 weeks postoperatively. In a study by Forrest (1992), seen in 27% and required an average of 5 days of parenteral narcotic analgesics [26]. In our study postoperative pain was seen in 5% of cases and required parenteral anti-inflammatory analgesics for 4 days then starting with oral analgesics after that.

Anatomically, bone harvest from anterior iliac crest via lateral approach would be expected to have more postoperative pain and gait disturbance [27-30].

However, the results of our study support Tagapongsa study in 1994 in that there is no difference in medial and lateral approaches [31]. Because the cancellous bone reservoir is located in the iliac crest area at the anterior 1/3 between the anterior iliac spine and tubercle, reflection of tensor fascia, gluteus medius, and gluteus minimus muscle in the lateral approach and iliacus muscle in the medial approach is inevitable. A large hematoma or excessive trauma can cause psoas muscle inflammation or dysfunction leading to postoperative pain and gait disturbance from anterior iliac bone harvest, injury to these muscles and bleeding from the cancellous marrow must be reduced to minimum [32].

When mucous membrane was intact or only slightly damaged, grafting was practical proposition, but quite contraindicated when the mucosal laceration were ragged or difficult to close. From reviewing the cases in our study, we had one case of failure due to penetration of mucous membrane by the bone graft in maxilla. We believe that watertight suture not under tension of the mucosal incision to prevent leakage of saliva with irrigation of the mouth by antiseptic wash will help to prevent infection from oral microorganisms [29,30].

Introduction of nasogastric feeding tube may be a protective appliance for a period of time until complete healing of the mucosa observed. Low grade inflammation due to allergic reaction may be associated with the use of Dacron-urethane osteomesh with collection of serous fluid. This induces low-grade infection, which is a potential predisposing factor for bone resorption ended with infection and failure. We observe it in one case [31,32].

We concluded from our study that the preparation of the recipient site prior to bone grafting procedure is important for successful results, delayed reconstruction of facial bones after trauma is indicated because most wounds are contaminated with loss of tissues, immediate reconstruction of the mandible after resection of benign tumors with low recurrent rates is important to prevent collapse of the segments,

cortico-cancellous bone graft obtained from the iliac crest provides a suitable amount of bone with osteogenic potential which can be fixated to the bones with a simple and rigid fixation, we also obtained approximately normal looking contour of the defect sites. Our success rate was 85 %. In our study if we analyze the causes of failure in the three cases (15 %) we can say that for the mandible the cause of failure is due to inflammation due to the osteomesh which is regarded as a foreign body (Fig. 4). For the alveolar cleft the cause probably was due to the use of only cancellous bone without any means of fixation and breakdown ofone of the sutures exposing the grafts and causing infection and therefore became a sequestra acting as a foreign body, therefore we suggest using a cortical bone graft and fixed with a micro-plates then we insert a cancellous bone and a water tight seal closure and instruction about good oral hygiene with chlorehexidine mouth wash. For zygomatic bone graft the cause might be due to infection by a maxillary sinus communication that might occur later.

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Correspondence to Dr. Ayad AHasan
E-mail:ayadoo2000@yahoo.com
Mobile 07901755618
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Evaluation of Immunohistochemical Expression of CK19 in Papillary Thyroid Carcinoma and Grave's Disease with Papillary Changes

Ikram A. Hasan[1] *FICMS*, **Methaq M. Najem**[1] *MSc*, **Thair J. Kadem**[2] *FICMS*
[1]Dept. of Pathology, College of Medicine, Al-Mustansirya University, [2]Al-Yarmook Hospital, Baghdad, Iraq.

Abstract

Background Immunohistochemistry seems to be important method for differentiation between papillary thyroid carcinoma which is the commonest thyroid cancer and Grave's disease which may be challenging by light microscopic features only.

Objectives The aim of the study was to evaluate the immunohistochemical expression of CK 19 antibody that is considered a marker of malignancy in papillary thyroid carcinoma and in Grave's disease and to determine whether CK19 is effective in the discrimination between these two pathological conditions.

Methods In this retrospective study paraffin embedded archival materials from 40 cases including 20 papillary thyroid carcinoma and 10 Grave's disease in addition to ten normal thyroid tissue were used as a control group, collected from the department of pathology of Al-Yarmook Teaching Hospital for the period between Jan 2009 to Jan 2011. The immunohistochemical expression of CK19 markers was assessed for intensity and extent of staining in semiquantative method.

Results Sixteen of the 20 papillary carcinomas showed diffuse and intense cytoplasmic staining with CK19 (80%), 4 cases showing diffuse faint staining (20%). Seven of the 10 Grave's cases (70%) are completely negative. The remaining 3 cases showing focal weak staining with CK19 (30%). There was a significant difference in the extent of staining between papillary thyroid carcinoma and Grave's disease and there was highly significant difference in intensity of staining between them.

Conclusions The staining features of CK19 may be helpful in the differential diagnosis between papillary carcinoma and Grave's disease with papillary carcinoma like structures. This immunoreactivity should be evaluated with histopathological findings in order to prevent over diagnosis of papillary carcinoma.

Keywords CK19 immunohistochemical expression, papillary thyroid carcinoma, Grave's disease.

Introduction

Papillary thyroid carcinoma is the commonest thyroid cancer [(1)] and there is a marked increase in its incidence through the recent decades [(2)].

The identification of papillary thyroid carcinoma relied on the presence of papillary architecture. The current accepted diagnosis of this entity is based on nuclear features that include optical clearing, elongation, overlapping and irregular contours with grooves and pseudoinclusions [(3)]. However, identification of these features remains at times controversial and the distinction of papillary carcinoma from other benign thyroid lesions with papillary features can be difficult.

One of these benign lesions is the autoimmune hyperthyroidism (Grave's disease) that is predominantly seen in females. In Grave's

disease, the thyroid is generally diffusely enlarged, but there may be nodules as well [5].

The thyroid in Graves' disease may contain foci showing papillary formation microfollicles, vesicular nuclei, and nuclear grooves, and it may be hard to distinguish these foci from papillary carcinoma depending only on microscopic features [5]. This difficulty may also be encountered in papillary formations of multinodular goiter, where CK19 has been shown to be effective in discrimination [6].

Because of these problems, numerous attempts have been made to apply a variety of techniques to enhance diagnostic reliability like electron microscopy and flow cytometry [7] but the results were disappointing in that the techniques did not yield cheap and rapid diagnostic information that could realistically change the practice of surgical pathology. Over the past decade, however, rapidly expanding techniques available in molecular pathology (like immune-histochemistry) have begun to show real promise to change daily practice and many immunohistochemical markers have been evaluated for their potential in distinguishing Papillary thyroid carcinoma from other benign thyroid lesions, the main ones including cytokeratin (CK) 19, galectin-3 (GAL3), and HBME1 (17).

Cytokeratin polypeptide 19 (CK19) is a type I intermediate filament protein and is the smallest known keratin and is remarkable in that, contrary to all other keratins, it does not have a designated partner for the formation of filaments implying that regulation of its expression is different from other keratin-encoding genes [8]. Cytokeratin 19 concentrates at sarcomeres of striated muscle and copurify with the dystrophinglycoprotein complex, perhaps through the interaction of the cytokeratin with the actin-binding domain of dystrophin. In vitro studies showed that dystrophin binds directly and specifically to CK19 [9]. CK19 is synthesized in simple and stratified epithelia.

This study was designed to determine the effectiveness of CK19 in distinguishing papillary thyroid carcinoma and papillary carcinoma-like changes in Graves's disease.

Methods

Tissue Sample: In this retrospective study a total of 30 tissue samples of which 20 were of papillary thyroid carcinoma and 10 of Grave's disease. In addition, 10 normal thyroid tissue had been taken as a control. All formalin fixed, paraffin-embedded tissues were retrieved from the archived files of the department" of histopathology of Al-Yarmook Teaching Hospital for the period between Jan 2009- Jan 2011.

Clinicopathological parameters (age, sex, clinical presentation and histopathological diagnosis) were obtained from the available histological reports. For each case, 2 sections of 5μm thickness were taken; one section was stained with (H and E), and the other was stained immunohistochemically for with CK 19 tumor markers.

Immunohistochemical staining was performed by the streptavidin –biotin method.

Interpretation of the results of staining characteristics:

The presence of brown reaction product of more than 10% of tumor cells at the site of the target antigen is indicative of positive reactivity. Counter stain will be pale to dark blue coloration of the cell nuclei. Staining pattern was cytoplasmic or membrane and cytoplasm.

The quantity of the immunostaining was evaluated as follows [10]: Semiquantitative evaluations were made on the basis of intensity and extent of staining for CK-19. The extent of staining by CK-19, were calculated according to the percentage of positive cells as: No staining: 0; < 25% stained cells: 1+; 25% to 75% stained cells: 2+; > 75% stained cells: 3+, whereas the intensity of staining by CK-19 was evaluated as no staining: 0; faint: 1+; and strong: 2+.

The positive result was classified as focal and diffuse. Focal:-tumors in which clusters of positive cells where seen in some areas of the tumor but other region where negative. Diffuse: - tumors in which isolated and/or clusters of

positive cells where seen throughout most areas of the tumor.

Results

The papillary carcinoma group consist of 19 females and 1 male with an age range 25-55 years, Grave's group comprise 8 females and two males with an age range 18-60 years (Table 1).

Table 1. Age distribution of patients with papillary carcinoma and Grave's disease.

Age (years)	Papillary carcinoma		Graves	
	No.	%	No.	%
<50	12	60	8	80
≥50	6	40	2	20

Sixteen out of twenty cases (80%) of papillary carcinomas showed diffuse and intense cytoplasmic staining with CK19 (Fig.1 and 2), four cases (20%) showing diffuse faint staining. Seven of the 10 Grave's cases (70%) are completely negative (Fig.3 and 4). The remaining 3 cases (30%) showing focal weak staining with CK19.

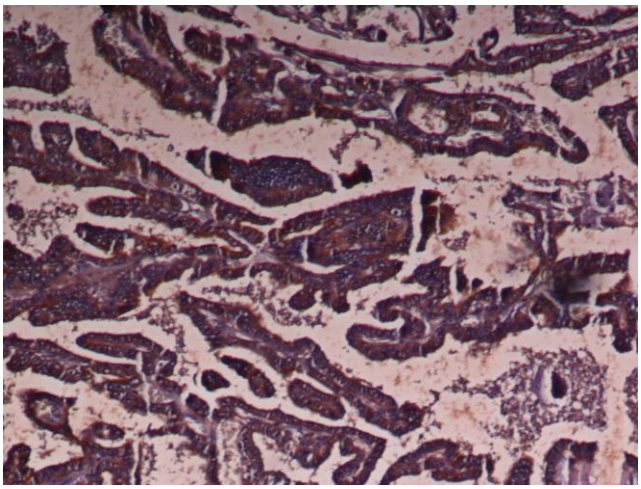

Fig. 1. Diffuse, strong CK19 staining in papillary carcinoma (CK19X100)

There was a significant difference in the extent of staining between papillary thyroid carcinoma and Grave's disease (P = 0.005) by using Chi square test and there was highly significant difference in intensity of staining between them (P = 0.0001) (Table 2 and 3).

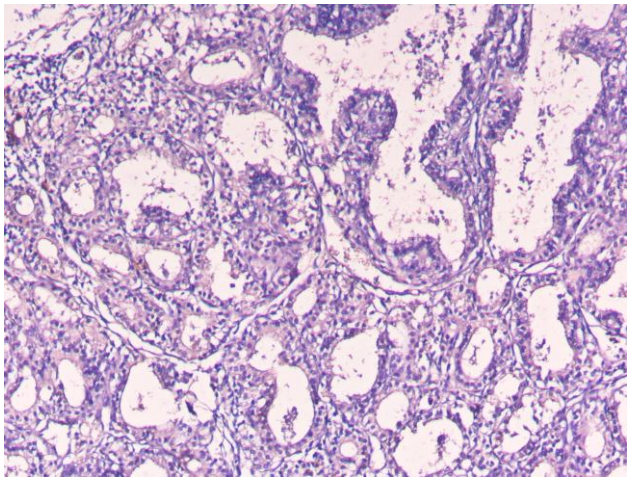

Fig. 2. Diffuse, strong CK19 staining in papillary carcinoma with strong desmoplasia, CK19X100

Discussion

One of the most frequent problems in thyroid pathology is differentiating papillary carcinoma like changes in Grave's disease which may contain vesicular nuclei, nuclear grooves from true papillary carcinoma which may be difficult depending on microscopical features only so there is a need for other methods like immunohistochemistry to solve this problem using different markers like (HMB-1, galectin-3 and CK19).

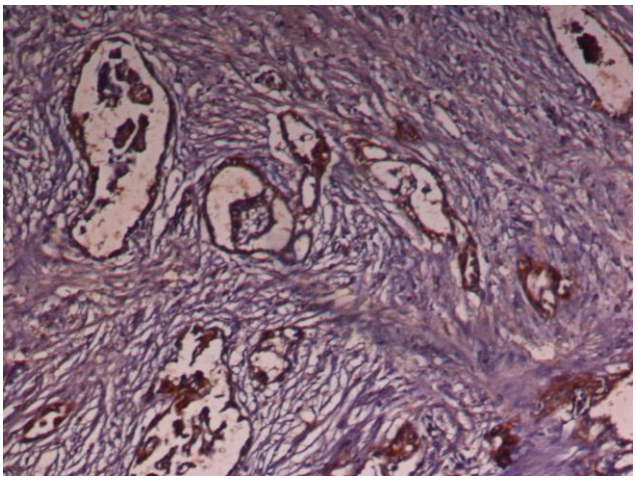

Fig. 3. Faint or absent staining with CK19 in Grave's disease (CK19X100)

CK19 is one of the low molecular weight keratin. It is known to be resistant to denaturation and the preservation of its reactivity has been reported even in necrotic tumor tissue [11].

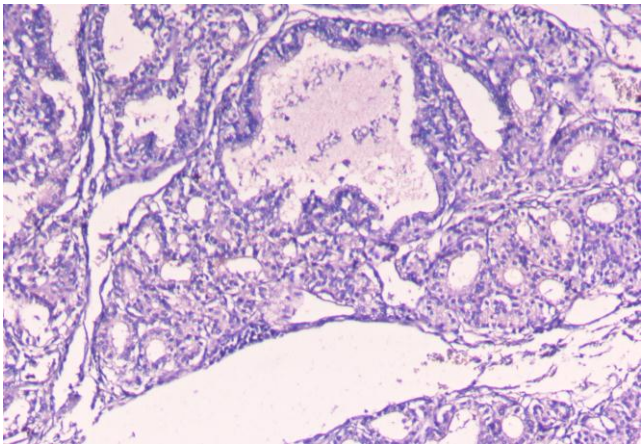

Fig. 4. Another view showing faint or absent staining with CK19 in Grave's disease (CK19, X100)

Diffuse and strong staining with low molecular weight keratins has been reported in thyroid papillary carcinoma [12,13].

In this study, 16 of the papillary carcinoma group exhibit diffuse intense cytoplasmic staining for CK19 and seven of ten Grave's cases are completely negative while the three remaining cases showed diffuse, faint immunoreactivity.

The results of current study agree with other studies like Suna et al [5] that demonstrated that the vast majority of papillary carcinoma exhibit diffuse intense cytoplasmic staining for CK19 whereas majority of Grave's cases are completely negative.

Table 2. Extent of staining of CK19 in papillary carcinoma and Grave's disease.

Extent	Papillary carcinoma		Graves	
	No	%	No	%
0	1	5	6	60
+1	1	5	2	20
+2	3	15	2	20
+3	15	75	0	0

P = 0.005

The weak immunoreactivity in Grave's disease may be similar to the pale staining reported previously by Sahoo et al and Bennet et al in follicular adenoma [1,14].

Table 3. Intensity of staining of CK19 in papillary carcinoma and Grave's disease.

Extent	Papillary carcinoma		Graves	
	No	%	No	%
0	1	5	8	80
+1	22	10	2	20
+2	17	85	0	0

P = 0.0001

The results of this study is in agreement with others like: El Demallowy et al [2] who showed that 85% of cases of papillary carcinoma were positive for CK19 and with Cheung et al [3] who reported diffuse CK19 staining in 80% of papillary thyroid carcinoma and with Baloch and Coworkers [15] who showed that all cases of papillary thyroid carcinoma were positive for CK19 and with Shin et al [16] who showed that 80-90% of cases were positive for CK19 and with Theresa et al [17] who showed that 96% of cases were positive for CK19.

Benign thyroid lesions such as follicular adenomas and multinodular goiter with papillary formations are generally negative for CK19, but may sometimes show faint staining.

This low molecular weight cytokeratin has also proved to be effective in discriminating papillary carcinoma from multinodular goiter exhibiting papillary formation and follicular adenoma [6]. Focal and pale staining with CK19 may be seen in follicular adenoma and multinodular goiter with papillary formation [18]. The vast majority of cases of follicular adenomas exhibit no or focal staining with CK19 [1,5,14]. CK19 immuno-histochemical staining revealed no or focal expression in the majority of cases of papillary hyperplasia [19,20].

In conclusion the staining features of CK19 may be helpful in the differential diagnosis between papillary carcinoma and Grave's disease with papillary carcinoma like structures. This

immunoreactivity should be evaluated with histopathological findings in order to prevent over diagnosis of papillary carcinoma. Other tumor markers had been studied in Iraqi patients with thyroid carcinoma like estradiol, progesterone [21] and tumor suppressor gene P53 [22].

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Correspondence to Dr. Ikram A. Hasan
E-mail: Ikram_alhadithi@yahoo.com
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The Influence of N-RAS Gene Mutations on the Response to Induction Therapy in AML Iraqi Patients

Nahidh K. Alwan[1] *MBChB MSc*, **Raad J. Musa**[2] *MBChB PhD*, **Ban A. Abdul-Majeed**[2] *MBChB PhD*

[1]Hematology Lab. Al-Imamian Al-Kadhimiyian Medical City, [2]Dept. of Pathology & Forensic Medicine, Collage of Medicine, Al-Nahrain University

Abstract

Background N-RAS mutations are the most commonly detected molecular abnormalities in hematologic malignancies, especially in those of myeloid origin.

Objective Current study aimed to determine the frequency of N-RAS mutation; and its influence on response to induction therapy in patients with acute myelogenous leukemia (AML) in Iraq.

Methods Peripheral blood and bone marrow samples were taken from 58 newly diagnosed AML patients and 30 individuals with reactive bone marrow were selected as a control group. Samples screened for N-RAS gene mutations using nested PCR were followed by mutation sensitive digestion analysis (MSDA).

Results N-RAS mutations at the time of diagnosis were found in 10/58 (17.24%) patients with AML and no mutation in control individuals. Patients with mutant N-RAS showed lower complete remission (CR) than wild type, the difference was not significant (60% vs. 72.92%, $P = 0.414$).

Conclusion The current results provide clues for activation of RAS-signaling cascade in AML patients, supporting their role in molecular pathogenesis of leukemia. N-RAS mutations show no influence on CR rate in AML patients. Further studies on larger scale to define the prognostic significance of N-RAS mutations are recommended.

Keyword AML, N-RAS mutation, MSDA, complete remission.

Introduction

RAS proteins are small GTPases that act as molecular switches, transducing extracellular signals from activated receptors at the cell surface to the nucleus, thus, regulating cell proliferation, survival, and differentiation. Three RAS genes encode 4 widely expressed isoforms: H-RAS, N-RAS, and the splice variants K-RAS4A and K-RAS4B [(1)].

The RAS proteins possess intrinsic GTPase activity (induced hydrolysis of GTP to GDP), which normally leads to their inactivation and the control signal transduction. In tumors, a point mutation resulting in loss of the intrinsic GTPase activity and RAS proteins lock in an active state, does not stop anymore to send signal stimulating cell proliferation and appears to be associated with transforming activity of the protein. All RAS mutations were missense point mutations occur at codons 12, 13 (exon 1) and 61 (exon 2) [(2,3)]. Activating mutations of N-RAS are most common among myeloid malignancies, found in approximately 20% to 40% of myelogenous leukemia (AML), myelodysplastic syndrome (MDS), chronic

myelomonocytic leukemia (CML) and juvenile myelomonocytic leukemia (JMML) [1].

AMLis characterized by a maturation block and accumulation of myeloid progenitor cells. Clinically, cytogenetically, and molecularly it has been recognized as a heterogeneous disorder [4]. Although in AML N-RAS mutations were first reported 25 year ago, the prognostic impact of N-RAS mutations is still under discussion and seems to vary from disease to disease. Several studies indicate an association with poor outcome; others found a negative prognostic impact of N-RAS mutations only in AML with favorable karyotypes; other found N-RAS mutations associated with a favorable prognosis and at last some studies could not defined a prognostic impact of N-RAS mutations [5]. The current study aimed to determine the frequency of N-RAS mutation, its influence on response to induction therapy in AML patients in Iraq.

Method

Fifty eight newly diagnosed untreated AML patients and thirty individuals with reactive bone marrow (including 19 individuals presented with pyrexia of unknown origin and 11 presented with idiopathic thrombocytopenic purpura) served as control group were enrolled in thisstudy at Department of Hematology / Baghdad Hospital at Baghdad Medical City for the period April 2011 to July 2012.

The study was approved by the Ethics Committee of College of Medicine, Al-Nahrain University and informed consent in accordance with the Declaration of Helsinki was obtained from patients, control individuals or their legal guardians prior to the collection of samples and data.

DNA extraction from peripheral blood, amplification and enzyme restriction was done at the Department of Pathology/Baghdad Collage of Medicine. Genomic DNA was extracted from peripheral blood specimens of patients at time of presentation, N-RAS gene amplificationperformed; briefly 1 µL of the extracted DNA was added to a 20µL PCR reaction mixturecontaining 5 µL of AccuPower TLA PCR Premix, 10 pmol of each forward and reverse primer (Table 1) and 13µL of nuclease free water. The first round of PCR consisted of 30 cycles (denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, and extension at 72^{o}C for 30 seconds). 1 µL of the amplified product of the first round was then added to a second 20-µL PCR reaction mixture using 2nd set of primers for a further 30 cycles under identical conditions to the first round. Each round was preceded by heating at 95^{o}C for 10 minutes. Negative control (no DNA template) tube was included with each batch of samples analyzed. Beta globulin gene also amplified as control for amplification [6].

Table 1. Sequences of DNA primers

First Round N-RAS Gene Primers: [6]
• RS 12 (Forward) 5’ GCTCGCAATTAACCCTGATTAC • RS7 (Reverse) 5’ ATTCCTTTAATACAGAATATGG
Second Round N-RAS Gene Primers: [6]
• RS6 (Forward) 5’ACTGAGTACAAACTGGTGGTGGTTGGACCA • RS5 (Reverse) 5’ GGTCAGCGGGCTACCCCTGGACCA

Mutation sensitive digestion analysis (MSDA) was used for detection of mutations at codon 12 and codon 13. The second round PCR primers (RS6 and RS5) are both mismatched at a single base from their target sequence. This creates a 5’ *Bst*NI restriction site at codon 12 and 3’ restriction site within sequence at the downstream end of amplified DNA. If the amplified DNA has normal sequence at the first two bases of codon 12, it is cleaved at both the 5’ and 3’ sites by *Bst*NI to produce an 87 bp fragment, whereas mutant DNA with a

substitution affecting either of the first two bases at codon 12 results in loss of this restriction site and thus cleaves only at the 3' site to produce a 116 bp fragment. A codon 13 mutation creates an *Hph*I recognition site. Digestion of the 135 bp amplified fragment with this enzyme thus leads to cleavage of mutant DNA at a 5' and a 3' site to produce a 75 bp fragment, while normal sequence is digested at only the 3' position to produce a fragment of 117 bp. For both enzymes, the 3' site is always cleaved and serves as a control for the digestion (Figure 1) [6].

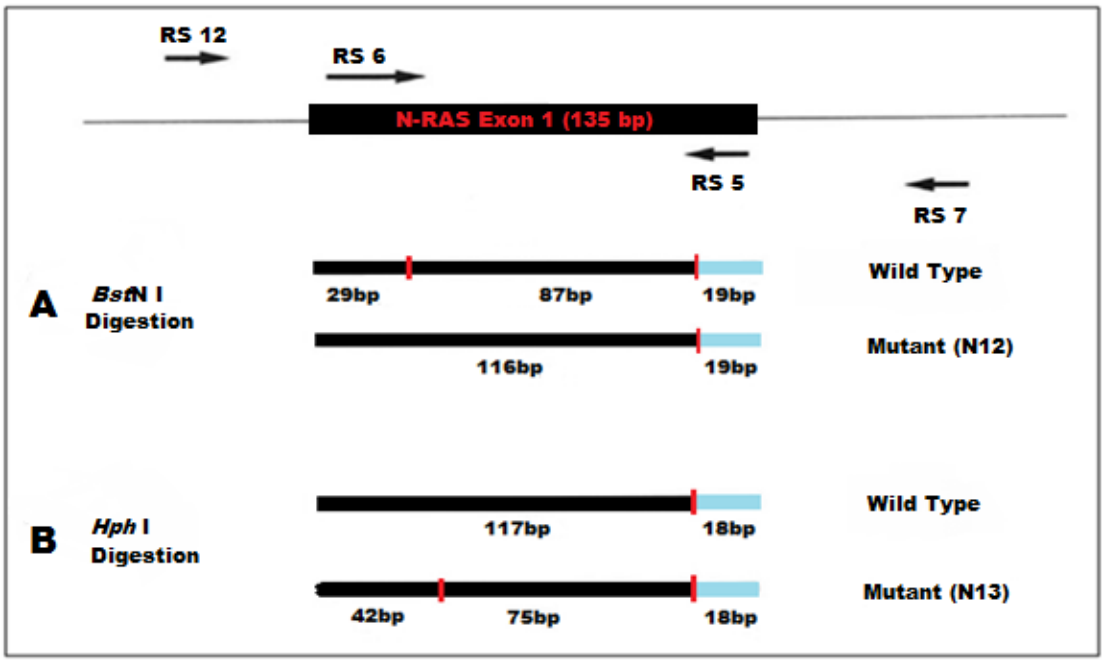

Fig. 1. Schematic illustration of the PCR-based MSDA used for the detection of codon 12 and 13 mutations. The positions of the first round primers for exon 1 (RS12 and RS7) and the second round nested primers (RS6 and RS5) are shown. (A) *Bst*NI digestion of amplified sequence for codon 12 mutations. (B) *Hph*I digestion of amplified DNA for codon 13 asp mutations [6].

PCR products were digested directly after amplification, for codon N12 detection, 10 µl of PCR reaction mixture (about 0.1-0.5 µg of DNA), 7 µl of nuclease free water, 2 µl of NE Buffer 2 (10X) and 1µlof *Bst*NI were mixed gently for a few seconds. Then incubated at 60°C for 2 hours and mixture were subjected to electrophoresis in 2% agarose gels containing ethidium bromide. For codon N13 detection, 10 µl of PCR reaction mixture, 7 µl of nuclease free water, 2 µl of NEBuffer 4 (10X) and 1µl of *Hph*I were mixed gently for a few seconds. Then incubated at 37°C for 2 hours and mixture were subjected to electrophoresis in 2% agarose gels containing ethidium bromide (Figure 2, 3 and 4) [6,7].

Induction Therapy

The primary objective in treating patients with AML is to induce remission and thereafter prevent relapse. Complete remission (CR) was defined morphologically as no circulatory blasts, with absolute neutrophil count (ANC) $>1.5\times10^9$/L, and platelet count $>100\times10^9$/Land cellular marrow with blasts <5% and absence of extra medullary involvement [8]. Failure of induction was defined as less than 50% reduction in marrow blast percent from that at presentation.Patients neither in complete remission nor in failure regarded as partial response. Treatment is conventionally divided into two phases: induction and post induction [8].

Doxorubicin 30mg/m^2 per day i.v. infusion over 30 min from day 1-3 and Cytosine Arabinoside 100mg/m^2 per day i.v. infusion over 16 hours from day 1-7. For FAB group M3, doxorubicin 30mg/m^2per day i.v. infusion over 30 min was given on days 1, 3, 5, 7 (4 doses) along with All Trans Retinoic Acid (ATRA) in a dose of 45mg/m^2 per oral daily in 2 divided doses from day 1 till remission[9].

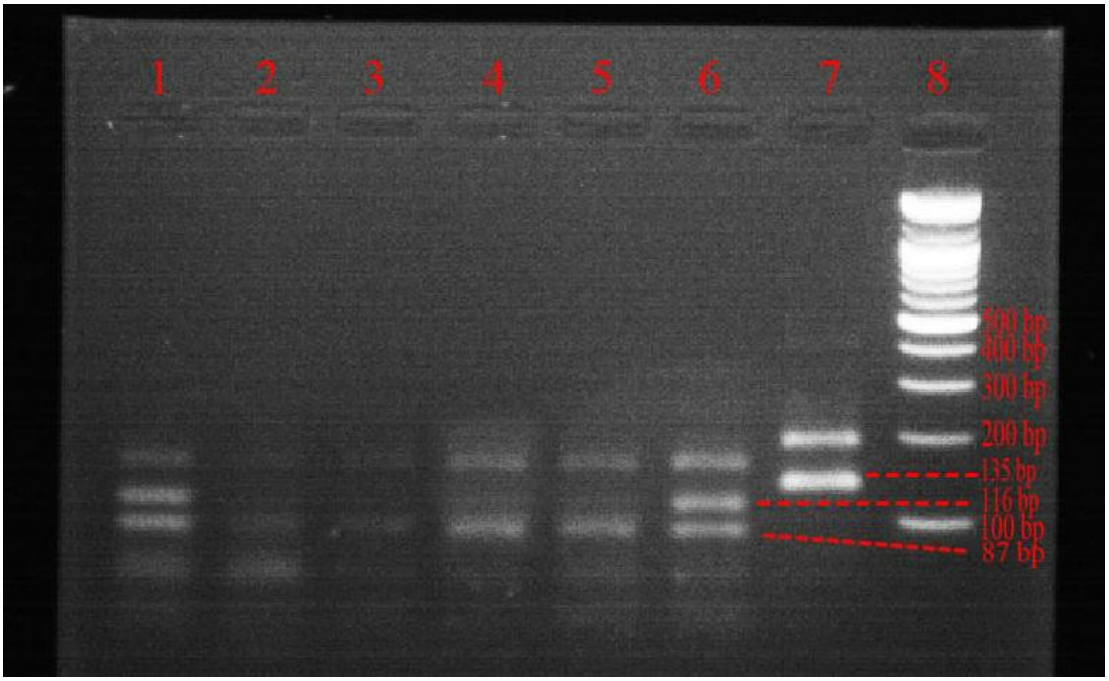

Fig. 2. Mutation sensitive digestion analysis (MSDA) from AML patients. PCR amplified DNA digested with BstNI for N-RAS codon 12 mutation. Lane 7, undigested control; lane 2, 3, 4 and 5 were wild N-RAS AML patients; Lanes 1 and 6 show AML cases with mutant N-RAS AML patents (116-bp band in lane 1, 6 was a result of N-RAS N12 mutation); lane 8, DNA size markers. Electrophoresis was done in 2% agarose gel containing ethidium bromide (final concentration 0.5 µg/ml) at (4V/cm) for 60 min.

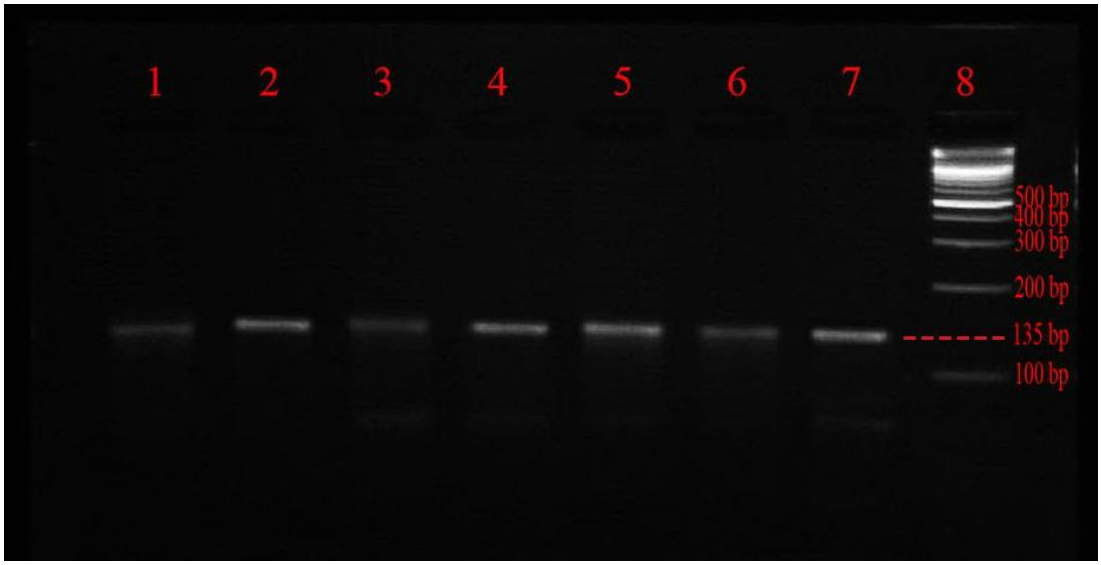

Fig. 3. Mutation sensitive digestion analysis (MSDA) from AML patients. PCR amplified DNA digested with HphI for N-RAS codon 13 mutation. Lane 7, undigested control; lane 1, 2, 3, 4, 5 and 6 were wild N-RAS AML patients; Lane 8, DNA size markers.*Electrophoresis was done in 2% agarose gel containing ethidium bromide (final concentration 0.5 µg/ml) at*(4V/cm) *for 60 min.*

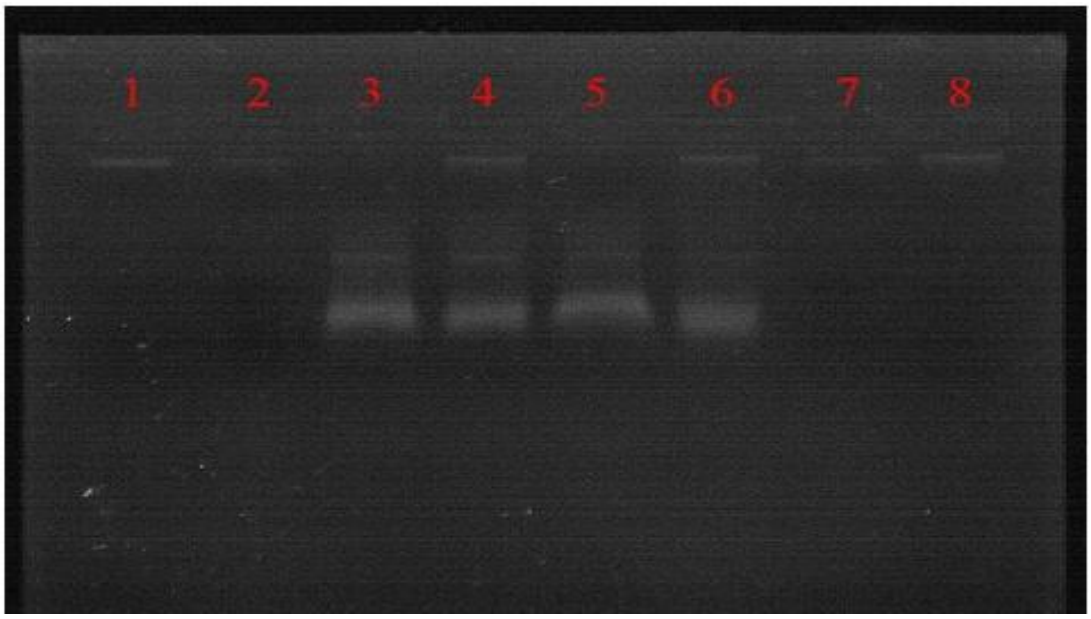

Fig. 4. BstNI and HphI enzymes digestion control (lanes 1, 3, 5 and 7 were unamplified DNA from AML patients while lanes 2, 4, 6 and 8 were unamplified DNA from control individuals). Lanes 3 and 4 contain DNA digested with BstNI. Lanes 5 and 6 contain DNA digested with HphI. Digested lanes show smear in comparison with undigested lanes which show single bands. Electrophoresis was done in 2% agarose gel containing ethidium bromide (final concentration 0.5 µg/ml) at (4V/cm) for 60 min.

AML patients pathology reports were retrieved again from archive of Department of Hematology/Teaching Laboratories at Baghdad Medical City) near the end of the study in order to assess patients' response to induction therapy (in term of complete remission, partial remission or failure) after 2-3 weeks from induction.

Data were analyzed using SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as (mean ± SE) and frequency was used to express discrete data. Student T-test was used to analyze numeric data while Chi-square and test was used to analyze discrete data. Values were considered statistically significant when ($P < 0.05$).

Results

Out of 58 patients with AML, there were 33 (56.89%) males and 25 (43.10%) females with a M:F ratio 1.3:1, mean age was 41.57 ± 2.53 year (age range was 13-75). Out of 30 individuals in control group, there were 18 (60%) males and 12 (40%) females with a M:F ratio 1.5:1, mean age was 38.77 ± 2.93 year (age range was 16-70).

N-RAS mutations were found in 10 out of 58 (17.24%) of AML patients ($P = 0.091$). All mutations were in codon 12 and no mutation in codon 13. No mutations were detected in control group.

There was no significant difference in patient's gender ($P = 0.855$) and mean age between mutant and wild type N-RAS AML patients (40.2 vs. 41.85, $P = 0.407$). The mean WBC count was significantly higher (54.33 vs. 31.25 x 10^9/L, $P = 0.033$) and the mean bone marrow blast percentage was significantly lower (56.50 vs. 69.31%, $P = 0.025$) in patients with mutated N-RAS than that of patients with wild type N-RAS. There was no significant difference in N-RAS mutation among different AML FAB subtype ($P = 0.105$) (rest of results summarized in table 2 and 3).

Regarding response to induction therapy, forty eight (82.76%) patients had received 3 and 7 induction regimen while ten (17.24%) patients with AML-M3 received ATRA as induction regimen.

Table 2. Correlation between N-RAS mutation and clinical-hematological parameters

Parameter		Mutant N-RAS N = 10	Wild Type N-RAS N = 48	*P*
Gender	Male	18.18%	81.82%	0.828
	Female	16%	84%	
Age (Mean ± SE)		40.20 ± 6.27 year	41.85 ± 2.80 year	0.407
WBC (Mean ± SE)		54.33 ± 9.19 x 10^9/L	31.25 ± 7.64 x 10^9/L.	**0.033**
Hematocrit Percentage (Mean ± SE)		24.50 ± 1.36 %	26.02 ± 0.95 %	0.185
Platelets Count (Mean ± SE)		45.80 ± 15.24 x 10^9/L	47.42 ± 8.12 x 10^9/L	0.463
BM Blast Percentage(Mean ± SE)		56.50 ± 5.12 %	69.31 ± 3.68 %	**0.025**
PB Blast Percentage(Mean ± SE)		38.50 ± 3.74 %	51.23 ± 5.10 %	**0.028**
Complete Remission (CR)		60%	72.92%	0.414
Anemia		80%	58.33%	0.199
Bleeding Tendency		40%	41.67%	0.922
Fever		40%	45.83%	0.736
Weight Loss		30%	8.33%	0.056
Splenomegaly		50%	33.33%	0.318
Hepatomegaly		40%	22.92%	0.262
Lymphadenopathy		20%	18.75%	0.927

BM = bone marrow, PB = peripheral blood

Table 3. Distribution of N-RAS mutations within AML subtype according to FAB classification

FAB Classification	Mutant N-RAS		Wild N-RAS		Number of Cases	*P* value
	No.	%	No.	%		
AML-M0	1	16.67	5	83.33	6	0.969
AML-M1	2	18.18	9	81.82	11	0.926
AML-M2	4	19.05	17	80.95	21	0.784
AML-M3	1	10.00	9	90.00	10	0.837
AML-M4	1	25.00	3	75.00	4	0.67
AML-M5	1	20.00	4	80.00	5	0.864
AML-M6	0	0.00	1	100.00	1	0.605
Total	10	17.24 %	48	82.76 %	58	0.105

Thirty five (60.34%) patients achieved CR, six (10.34%) patients achieved partial remission, fourteen (24.14%) patients failed to achieve CR and there was no data available about 3 (5.17%) patients.Five (50.0%) out of 10 AML patients with mutant N-RAS and 30 (62.5%) out of 48 AML patients with wild type N-RAS achieved CR. One (10.0%) out of 10 AML patients with mutant N-RAS and 5 (10.42%) out of 48 AML patients with wild type N-RAS achieved partial remission. Four (40.0%) out of 10 patients with mutant N-RAS and 10 (20.83%) out of 48 patients with wild type N-RAS failed to achieve CR. No data was available about 3 (6.25%) out of 48 patients with wild type N-RAS. There was no significant difference (*P* = 0.501) in CR rate between patients with mutant and wild type N-RAS.

Discussion

The clinical significance of RAS mutations has not been uniformly established.In current study, N-RAS gene mutations were found in 17.24% of patients with AML. This result confirm previous reports that recognized a frequency of N-RAS

mutations to be in between (9-21%) in patients with AML [2,5,11-13]. Discrepancy in RAS mutation frequency among various reports result from fact that criteria for selection of AML patients differ between various studies, N-RAS frequency in studies analyzed only denovo AML was lower than studies select AML that arose from proven MDS which is more frequently associated with N-RAS mutations [11]. Also the difference in RAS mutation frequency may explained by number of cases involved, method of screening, number of exon examined (codons 12, 13 in exon 1, codon 61 in exon 2) and type of RAS mutation (N, K and H-RAS) analyzed [13]. All N-RAS mutation detected in codon 12 (100%) and no mutation detected in codon 13, these finding were in agreement with previous studies [5,11-14].

Although that HphI enzyme digested the unamplified DNA, it failed to digest a 3' end of the amplified DNA (that served as a control for enzyme function). Current study suggested that this negative result is not due to failure of the primer system to detect mutations in the digested PCR product but the predominance of digestion resistant band as mentioned in previous report. Bashey and Todd studies describe an overrepresentation of the singly digested band, which is caused by the formation of restriction enzyme resistant hetero-duplexes between mutant and normal strands which are mismatched at a single base only [6,15]. In addition to that, the reverse Allele specific restriction analysis (ASRA) method described by Todd and Iland fails to demonstrate the presence or absence of wild type alleles, since a digestion resistant band merely indicates the lack of a specific mutation rather than the presence of wild type sequences [16].

Analyses revealed a statistically significant association between bone marrow blast percentage, WBC count and N-RAS mutation (P = 0.025, P = 0.033 respectively), however no significant differences had been found between the two groups with respect to age, gender, platelet count, hematocrit percentage and clinical outcomes. These findings were in agreement with those reported in previous literatures [2,5,11,14,17,18].

Mutation of the N-RAS gene affects the biology of AML. Transfection of various cell types with mutant RAS genes has been shown to stimulate secretion of interleukin-3, granulocyte, and granulocytemacrophage colony stimulating factors leading to autonomous growth through an autocrine mechanism, increasing peripheral WBC count [19].

The highest frequency of N-RAS mutation in M4 in current study corresponded with most of the previously published studiesm [5,11,13]. N-RAS mutation is most likely a postinitiation event contributing to the progression/proliferation of sub-clones in AML, selection and expansion of RAS mutant clones may provide a differentiative stimulus toward the monocytic lineage [2], Van Kamp study also suggested that N-RAS mutation preferentially influences hematopoiesis to myelomonocytic differentiation or myelo-monocytic cells are more susceptible for acquiring an N-RAS mutation since N-RAS mutations are more likely to develop in cells of myelomonocytic differentiation [20]. This may be consistent with the overrepresentation of RAS mutation in M4/M5 FAB types.

The low frequency of N-RAS mutation in M3 (10%) in current study corresponded with Bowen study, N-RAS mutation is relatively underrepresented in M3 where FLT3 ITD is overrepresented, both RAS mutation and FLT3 ITD are rarely present in the same tumor [2].

Current study showed that response to induction therapy was comparable to Alwan study and Lowenberg study were reported CR rate to be (70 - 80%) [8,9].

CR rate in mutant N-RAS patients was lower than wild type N-RAS patients, but the difference was not significant (P = 0.414). Published reports addressing the clinical significance of RAS mutations in patients with AML are inconclusive. Whereas some studies observed that the presence of N-RAS mutation did not significantly influence CR rate [2,15]. Others observed a significantly lower CR rate compared with patients without N-RAS mutation [12,21]. Third

group reported a beneficial clinical effect of RAS mutations in patients with AML in response to high dose cytarabine therapy (HiDAC) [22,23]. Last group did not show that patients with RAS mutations had significantly different outcomes [24]. This discrepancy between these studies findings may be explained by differences in the intensity of the chemotherapy protocols in use to treat group of patients and the number of cases analyzed [25].

In conclusion, N-RAS mutations show no influence on CR rate in AML patients. Further studies on larger scale to define the prognostic significance of N-RAS mutations were recommended.

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Correspondence to Dr. Nahidh K. Alwan
E-mail: nahidhkamel@yahoo.com
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Characteristics and Clinical Management of Female Patients with Fissure in Ano in Al-Kadhimiya City, Baghdad

Qahtan A. Mahdi *MBChB FICS*

Dept. of Surgery, College of Medicine, Al-Nahrain University

Abstract

Background Fissure in ano is a common painful anal problem in female patients.

Objective To study the sociodemographic variables of female patients with fissure in ano, and to identified the characteristic of anal fissure and their treatment among those patient in Al-Imamian Al-Kadhimiyian Medical City and two private hospitals.

Methods This is a prospective study that was carried out from May 2008 to May 2011. Two hundred fifty female patients with fissure in ano were interviewed regarding their age, marital state, address, level of education, clinical presentation and the subsequent management.

Result The commonest age of presentation in females was between 21-30 years. 78% of them were living at the peripheral areas of north of Baghdad, and 79.6% were of low level of education (primary school or below). The prevalence was found more in women with high parity especially when their child delivery was supervised by a midwife in their location. All patient has presented with anal pain, 55% were associated with bleeding per rectum and 64.2% with constipation. The location of fissures was 51.6% anterior, 48% posterior, 0.4% lateral in position. Less than half of patients underwent a surgical treatment. 77% of them were treated by lord dilatation under anesthesia and 23% by lateral sphinecteromty.

Conclusion Factors which had an impact on the clinical course and management of fissure in ano in female patients were found to be the social status, the level of education and the parity, therefore to prevent this illness, a cooperation between the heath services and the family health center and the government is necessary in order to overcome such a common problem.

Keywords Fissure in ano, Female

Introduction

Fissure in ano is a tear in the anoderm distal to the dentate line. The pathophysiology of the anal fissure is thought to be related to a trauma from either the passage of a hard stool or a prolonged diarrhea. A tear in the anoderm causes spasm of the internal anal sphincter which results in pain, increased tearing, and decreased blood supply to the anoderm. This cycle of pain, spasm, and ischemia contributes to development of a poorly healing wound that becomes a chronic fissure. The vast majority of anal fissures occur in the posterior midline, 10-15% occurs in the anterior midline and Less than 1% of fissures occur off midline [(1)].

The posterior anal canal is the most poorly perfused part of the anal canal. The delicate blood supply is further compromised, thus rending the posterior midline of the anal canal relatively ischemic. The fissure is just a tear in the anal mucosa and is defined as an acute anal

fissure. If the fissure persists over time; it progresses to chronic fissure that can be distinguished by its classic features. The fibers of internal anal sphincter are visible in the base of the chronic fissure and often an enlarged anal skin tag is present distal to the fissure and hypertrophied anal papilla are present in the anal canal proximal to the fissure [2]. The diagnosis is secured by the typical history of pain and bleeding with defecation, especially if associated with prior constipation and confirmed by inspection after gently parting the posterior anus. Digital as well as proctoscopic examination may trigger severe pain, interfering with the ability to visualize the ulcer. An endoscopic examination should be performed, but it can be delayed 4 to 6 weeks, until the pain is resolved with medical management or until surgery is performed for those cases refractory to medical therapy [3]. Local application of medications to relax the sphincter muscle, thus allowing the healing to proceed, was first proposed in 1994 with nitroglycerine ointment [4-7], and then calcium channel blockers in 1999 with nifedipine ointment [8,9], and the following year with topical diltiazem [10]. Branded preparations are now available of topical nitroglycerine ointment (Rectogesic (Rectiv) as 0.2% in Australia and 0.4% in UK and US) [11], topical nifedipine 0.3% with lidocaine 1.5% ointment (Antrolin in Italy since April 2004) and diltiazem 2% (Anoheal in UK, although still in Phase III development). A common side effect drawback of nitroglycerine ointment is headache, caused by systemic absorption of the drug, which limits patient acceptability.

A combined surgical and pharmacological treatment, administered by colorectal surgeons, is direct injection of botulinum toxin (Botox) into the anal sphincter to relax it. This treatment was first investigated in 1993. However it must be noted that, in many cases involving Botox injections the patients eventually had to choose another cure as the injections proved less and less potent, spending thousands of dollars in the meantime for a partial cure. Lateral sphincterotomy is the Gold Standard for curing this affliction [12]. Combination of medical therapies may offer up to 98% cure rates [13].

Surgical procedures are generally reserved for people with anal fissure who have tried medical therapy for at least one to three months and have not healed. It is not the first option in treatment.

The main concern with surgery is the development of anal incontinence. Anal incontinence can include inability to control gas, mild fecal soiling, or loss of solid stool. Some degree of incontinence can occur in up to 45 percent of patients in the immediate surgical recovery period. However, incontinence is rarely permanent and is usually mild. The risk should be discussed between the surgeon and patient.

Surgical treatment, under general anesthesia, was either anal stretch (Lord's operation) or lateral sphincterotomy where the internal anal sphincter muscle is incised. Both operations aim to decrease sphincter spasm and thereby restore normal blood supply to the anal mucosa. Surgical operations involve a general anesthetic and can be painful postoperatively. Anal stretch is also associated with anal incontinence in a small proportion of cases and thus sphincterotomy is the operation of choice [14].

Methods

This prospective study has been done for evaluation of 250 female patients with fissure in ano attending three hospitals in Baghdad: the general surgery clinic Al-Imamian Al-Kadhimiyian Medical City, Al-Thurgham Private Hospital and Al-Kadhimiya Private Hospital from May 2008 to May 2011. All of them were diagnosed clinically by taking a complete history and physical examination. The history included: patient's age, marital state, numbers of child births, the address, the occupation, type of fissure in ano and its location.

Results

One hundred (40%) female patients were aged between 21 and 30 year. The age range was 1 to 51 years. The highest percentage of women was married (76.8%). The highest percentage of

patient came from the periphery of Al-Kadhimiya (rural area) (78%), while the rest comes from Al-Kadhimiya city itself. The highest rate of patients was of low level of education (79.6%) while the rest were of high level of education (21.4%).

14.7% of cases were female who had delivered 1-3 children, 52.1% of cases had delivered 4-6 children while 33.2 % of patients had more than 6 children for last delivery.

About three quarters (73.6%) of cases had their child delivery at home (by a midwife) while only (26.4%) had delivered at hospital as shown in (Table 1).

Table 1. Demographic criteria

Criteria		N (%)
Age (years)	1 - 10	14 (5.6%)
	11 - 20	18 (7.2%)
	21 - 30	100 (40%)
	31 - 40	65 (25.4%)
	41 - 50	30 (12.4%)
	60	23 (9.4%)
Marital status	Married	192 (76.8%)
	Non – married	58 (23.2%)
Parity	1 - 3 children	28 (14.7%)
	4 - 6 children	99 (52.1%)
	6 children	63 (33.2%)
Place of delivery	Home	140 (73.6%)
	Hospital	50 (26.4%)
Educational status	Low education*	199 (79.6%)
	High education**	51 (21.4%)

* = primary school or below, ** = secondary school or above

The highest rate of patient suffered from fissure in ano for more than 5 years without seeking medical advice. The most common presenting symptoms is anal pain (100%), followed by constipation (64%), bleeding per rectum (55.2%), and abdominal pain (30.8 %).

During the local examination of all cases, it was found that fissure in ano was in an anterior location in (51.6%) of cases. The clinical type of fissure in ano was found chronic in (72%) of cases. Regarding the type of treatment; conservative treatment was used for the acute fissure (48%). For the chronic stage (52%) a surgical treatment was usually required, of these, (77%) had undergone lord dilatation under anesthesia while in (23%) of the surgically treated cases lateral internal sphinecterotomy was necessary as shown in (Table 3).

Table 2. Anal fissure clinical criteria

Criteria		N (%)
Duration	< 1 year	71 (28.4%)
	1 - 5 year	119 (47.6%)
	> 5 years	60 (24%)
Associated symptoms	Anal pain	250 (100%)
	Constipation	160 (64.2%)
	Bleeding Per-rectum	138 (55%)
	Abdominal Pain	77 (30.6%)
	Abdominal Distension	44 (9%)
Location of fissure	Anterior	129 (51.6%)
	Posterior	120 (48%)
	Lateral	1 (0.4%)
Fissure type	Acute	70 (28%)
	Chronic	180 (72%)
Treatment	Conservative	120 (48%)
	Surgical	130 (52%)
	Lord dilatation	100
	LIS	30

LAS = Lateral internal sphincterotomy

Discussion

Fissure in ano is a common painful anal problem affects females, more than males. The commonest age group, in this study, was between 21-30 year while in other studies that were done in Europe and USA, it was between 40-60 years of age [15,16,17]. This may be due to early marriage in our country.

The majority of cases (78%) of anal fissure were in females living in rural area. The same result was found in a study done in India, while in a study done in UK, the incidence was much different between the Urban and the rural areas [18,19,20].

The number of patient's children (parity) was found closely related to the prevalence of anal fissure in this study. In a study done in UK, the majority cases of fissure in ano occurred in the first and second delivery [21,22,23].

Delivery at home by a midwife is associated with a high rate of anal fissure (73.6%) which was also

higher than in other study done in Europe (52%) [24,16].

The social culture has great impact on the chronicity of the anal fissure in our society. Females are unlikely to consult a doctor during early appearance of the symptom. (47.6%) of cases had visited the doctor only after one year after symptoms appearance while (24%) after five years. In a study in Europe, (95%) of cases has visited their general practitioner in the earliest appearance of the symptoms, while only (0.1%) of cases did so after one year [25,26].

Anal pain was the commonest symptom in all cases, constipation (64%), bleeding per rectum (55.2%). This was similar when compared with a study result in Europe [27,28].

(51.6%) were anteriorly located fissures, (48%) posteriorly while (0.4%) were lateral. While in a study, (90%) were posteriorly and (10%) were interiorly located fissures [29].

In conclusions, many factors contribute to the occurrence of anal fissure among females such as social, educational and place of delivery. In order to overcome such problem, it is recommended to increase social awareness about such illness by health education through mass media especially for females during antennal care with emphasis on those with low education status from rural area. To provide good training for the midwives regarding safe measures during delivery of fetus to avoid development of anal fissure in the future.

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E-mail: qalg4@yahoo.com
Mobile: + 964 7902639128
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Predictors of Successful Urinary Stone Treatment by Extracorporeal Shockwave Lithotripsy

Uday H. Mohammad *FABMS*

Dept. of Surgery, Section of Urology, Mosul Medical College, Mosul University, Mosul, Iraq

Abstract

Background In spite of the recent advances in the endoscopic treatment of urinary stones, extracorporeal shock wave lithotripsy (ESWL) is still the treatment of choice for most renal and upper ureteric stones; however the outcome depends on multiple factors.

Objective To investigate the effects of stone density, as measured by Hounsfield Units (HU) by non-contrasted computerized tomography (CT), stone size and stone location on ESWL treatment outcome of urinary calculi.

Methods A prospective study that included 65 patients. Data collection and patient evaluation were performed in Al-Salam Teaching Hospital in Mosul, in the period from March 2012 to December 2012. Patients were submitted to clinical, biochemical and radiological assessments followed by ESWL treatment. Statistical analyses using chi-square, analysis of variance (ANOVA), correlation, regression were performed for statistical significance between ESWL treatment, stone fragmentation and stone density, size and location in the renal pelvicalyseal system.

Results ESWL success rate was high (92%) for low density stones (< 500 HU). ESWL treatment outcome and stone size were inversely related. CT stone densities of 700 HU or less were almost always successfully treated by ESWL. CT stone density and stone size combined account for nearly 74% of the variation in the number of shock waves required to attain fragmentation. Stones located in lower calyceal area had less success rates.

Conclusion Stone density measurement is helpful to predict the success of ESWL for urinary stones, stones with higher density, large size and lower location may better be managed by percutaneous nephrolithotomy or endoscopic procedures.

Key words CT stone density, ESWL, none contrasted CT scan (NCCT).

Introduction

In spite of the recent advances in the endoscopic treatment of urinary stones, extracorporeal shock wave lithotripsy (ESWL) is still the first mode of treatment for most renal and upper ureteric stones especially those with size range of 10-20 mm [(1)]. The success rate of this treatment modality is in the range of 60-90% in various series [(2-5)]. Different techniques have been used to determine the chemical composition of urinary calculi *in vivo* as it is considered a valuable factor determining the outcome of ESWL [(6)]. However, the outcome of ESWL treatment depends on many factors including; stone size, site, composition and the presence of obstruction or infection [(7)]. Nowadays, Non-Contrasted Computerized Tomography (NCCT) is the best diagnostic modality to evaluate renal colic, to distinguish radiolucent urinary stones from tumors or blood

clots and to diagnose renal calculi with high sensitivity and specificity of over 90% [8-13]. The ability of NCCT to detect density differences as low as 0.5% has been used to determine the composition and fragility of urinary stones, and hence the outcome of ESWL [14]. In previous studies, the NCCT attenuation value of urinary calculi has been investigated as a method to predict the outcome of ESWL for two main purposes: avoiding the extra medical costs associated with nonproductive exposure of renal tissue to ESWL sessions, and seeking alternative patient management strategies [15-17].

The objective of this study was to investigate the effects of stone density ((as measured by Hounsfield Units (HU) on NCCT)), stone size, and stone location on ESWL outcome and stone fragmentation of urinary calculi.

Methods

This is a prospective study that included 75 patients initially, however 10 patients were excluded due to elevated creatinine levels (more than 2 mg/dL), bleeding diathesis or obstructed kidney. Thus, the analyses, results and conclusions of this study were based on 65 patients who were prospectively followed at Al-Salam Teaching Hospital in Mosul from March 2012 to December 2012.

All 65 patients had initially undergone clinical, biochemical and radiological assessments before ESWL treatment sessions. Of the 65 patients, 38 were males (58%) and 27 were females (42%), mean age of 42 ± 17 years (17-76).

Urinary stone sizes ranged between 5-25 mm; of which 8 were located in the upper calyx, 9 in the mid calyx, 17 in the lower calyx, 24 in the renal pelvis and seven in the upper ureter. Fifteen patients had stone sizes less than or equal 10 mm, thirty patients had stone sizes of 11-20 mm, while the rest (20 patients) had stone sizes of 21-30 mm.

The maximal linear diameter of the stone was measured by NCCT scan. NCCT scan using contiguous three-millimeter section slices through the stone was performed and viewed on soft tissue setting (window width 350; window level 150 HU). Siemens Somatom Plus 4 scanner, at 120 kV and 206 mA, was used at a scan rate of one second per image. A pixel map of the largest region of interest within the stone was performed and consisted of 100 attenuation values in a 10 x 10 matrix; with each value on the pixel map representing the attenuation value for four pixels. The lowest, highest and most common attenuation values were recorded and the mean stone attenuation value was then calculated.

ESWLs of all patients were undertaken by the same staff using Siemens Electromagnetic Lithostar Multiline Lithotripter with fragmentation performed under fluoroscopic or ultrasonographic guidance.

A maximum of 2800 shock waves were delivered in each treatment session with maximum energy level of four. ESWL treatment was terminated if satisfactory fragmentation was noted earlier before delivering the maximum number of shocks (i.e., 2800) and before reaching the maximum number of ESWL sessions (i.e.,) 4 sessions.

Patients underwent plain x-ray or ultrasound 3 weeks after each ESWL session to determine if there is no stone fragmentation or if there are significant residual fragments (≥ 5 mm) which warrants another ESWL session.

The maximum number of ESWL sessions was 4 and the maximum duration of follow up was 12 weeks after which there is either complete stone clearance or failure of ESWL signifying failure of stone fragmentation or the presence of significant residual fragments (≥ 5 mm).

This failure of ESWL treatment indicates the need for another treatment option. Patients who achieved complete stone clearance underwent plain x-ray or ultrasound 6 weeks after treatment completion for final assessment of outcome.

In 16 patients with stones larger than 20 mm, or lower calyx stones larger than 15 mm, J.J. stent was inserted prior to ESWL. Thus the 65 patients were divided into two groups according to the outcomes of ESWLs.

The "success group" comprised patients who had successful stone fragmentation and subsequent stone clearance. The "failure group" comprised patients who failed to clear the stone because fragmentation either did not occur at all or did occur, but, with significant residual fragments (5 mm or larger in size).

Statistical analyses including chi-square, analysis of variance (ANOVA), correlation, regression and 95% confidence intervals were performed on the data to test the statistical significance of the various relationships between ESWL outcome and stone fragmentation on one side, stone density, size and location on the other side.

Results

The characteristics of both groups are shown in Table 1. The mean stone diameter of the failure group was marginally larger though statistically insignificant *(P* = 0.577). The mean stone density, of the failure group was nearly 60% larger than that of the success group; 1075 HU compared to 675 *(P* = 0.000). On average, the failure group had received 2.6 ESWL treatment sessions compared to only 1.4 sessions in the success group; a difference of nearly 86%. On average, nearly 7200 shock waves were delivered to the failure group compared to only nearly 4000 in the success group (both *P*-values = 0.000).

Table 1. Characteristics of ESWL treatment outcome groups

Variable	Variable mean and (standard deviation)			P Value
	Success group N = 46	Failure group N = 19	Both groups N = 65	
Age (years)	42.7 (17.4)	42.0 (17.3)	42.5 (17.2)	0.770
Stone diameter (mm)	18.3 (6.6)	18.8 (6.8)	18.4 (6.7)	0.577
CT Stone Density (Hounsfield units)	675 (285)	1075 (255)	785 (332)	<0.001
Number of ESWL treatment sessions	1.4 (0.7)	2.6 (0.9)	1.8 (0.9)	<0.001
No. of shock waves until fragmentation	4015 (1830)	7218 (2525)	4950 (2510)	<0.001

Stone Density

The patients were further analyzed by dividing them into three groups according to stone density. The "low density group" comprised all patients with stone densities of less than 500 HU, the "medium density group" comprised all patients with stone densities of 500-1000, while, the "high density group" comprised all patients with stone densities of more than 1000. ESWL treatment outcomes, according to stone density levels are shown in Table 2 showing high success rate in low density group (94%), A chi-square test analysis revealed statistically significant association between ESWL treatment outcome and stone density (chi-square = 12.4, df = 2, *P* = 0.002).

Table 2. ESWL treatment outcome according to CT stone density

CT stone density level (Hounsfield units)	Number of Patients (and %) with		
	Stone clearance (Success)	Non-stone clearance (Failure)	Total number
Low density group (< 500)	15 (94%)	1 (6%)	16 (100%)
Medium density group (500-1000)	22 (73%)	8 (27%)	30 (100%)
High density group (> 1000)	6 (32%)	13 (68%)	19 (100%)
Total	43 (66%)	22 (34%)	65 (100%)

Stone Size

The patients were also analyzed by dividing them into three groups according to stone diameter. The "low diameter group": stone diameters of 10 mm or less, the "medium diameter group": 11-20 mm, while, the "high diameter group": 21-30 mm. The ESWL treatment outcomes, in terms of success or failure of stone clearance, according to these three stone diameter levels are shown in Table 3. The success rates achieved were 93%, 73% and 45% for lower, medium and larger size groups respectively (chi-square = 6.8, df = 2, *P* = 0.032). A positive correlation between the stone diameter in millimeters and the number of shock waves delivered was noted r = 0.32, (*P* = 0.008).

Table 3. ESWL treatment outcome according to stone size

Stone diameter (Millimeters)	Number of Patients (and %) with		
	Stone clearance (Success)	Non-stone clearance (Failure)	Total number
Low diameter group (0-10 mm)	14 (93%)	1 (7%)	15 (100%)
Medium diameter group (11-20 mm)	22 (73%)	8 (27%)	30 (100%)
High diameter group (21-30)	9 (45%)	11 (55%)	20(100%)
Total	45 (69%)	20 (31%)	65 (100%)

Stone Site

Patients were stratified into two groups according to stone site; "lower calyceal group" included all patients with lower calyceal stones, and "other group" included the rest of patients. The ESWL treatment outcomes, in terms of success or failure of stone clearance, according to these two stone sites ("lower calyceal" or "other") are shown in Table 4. The success of ESWL treatment was only 35% in the lower calyceal stone site group compared to 75% in the case of other stone sites (chi-square = 6.3, df=1, P-value = 0.011). Regression analysis was also performed & it revealed that stone density alone accounts for nearly 70% of the variation in the number of shock waves required to attain fragmentation, while both, stone density and stone size combined, account for nearly 74% of the variation.

Table 4. ESWL treatment outcome according to stone site

Stone site	Number of Patients (and %) with		
	Stone clearance (Success)	Non-stone clearance (Failure)	Total number
Lower calyceal	6 (35%)	11 (65%)	17 (100%)
Other	36 (75%)	12 (25%)	48 (100%)
Total	42 (65%)	23 (35%)	65 (100%)

Our data also indicate that stone density in the success group is nearly 700 HU; indicating successful treatment by ESWL below this level and failure above 900 HU. The successful outcome was also observed with stone size of nearly 15.5 mm or less, with 1.7 maximum numbers of sessions and up to 4600 shock waves (Table 5).

Table 5. Means and 95% Probability Confidence Intervals

Variable		Mean	95% Confidence Interval	
			Lower bound	Upper bound
Stone density (Hounsfield units)	Success group	675	570	710
	Failure group	1075	905	1202
Stone Size (Diameter in millimeters)	Success group	18.3	15.6	20.2
	Failure group	18.8	15.7	22.5
Number of treatment sessions	Success group	1.4	1.2	1.7
	Failure group	2.6	2.2	2.9
Number of shock waves	Success group	4015	3412	4620
	Failure group	7218	6270	8160

Discussion

ESWL is still considered the best treatment for calculi less than 20 mm. The success rate is in the range of 60-90% in various series but the outcome of this therapy depends on different factors including stone composition, stone location, pelvicalyceal anatomy and stone size [15,17,18]. The success rate of ESWL for renal and upper ureteral calculi in Iraqi patients has been evaluated in some studies and is comparable to the other series, ranging between 60-85% and it is inversely related to stone size [3-5]. These studies described the effect of stone size on the success rate of ESWL but they didn't consider other factors as stone density and stone location in the urinary tract, therefore, further studies are needed to assess the effect of these factors on the success rate of ESWL in Iraqi patients. Stone composition seems to play the most important role in the outcome of treatment, however, still it cannot be known accurately before stone retrieval and analysis. The crystals excreted in urine after ESWL can give an idea about stone composition.

Plain x-ray has been used to predict the outcome of ESWL treatment by comparing stone density with bone density. However, this method has some disadvantages since the stone diameter and appearance might not be measured accurately, especially in the presence of bowel gas interference or neighboring bony structures and the density measurement is subjective [2]. In this study, we used plain CT scan which is a non invasive technique and provides greater density discrimination than plain x-ray. CT scan is more accurate in the evaluation of urinary stones [19]. It can distinguish density differences as low as 0.5% compared to only 5% discrimination using plain x-ray [2,7]. Recently, it is reported that the use of dual-energy multidetector CT can improve the detection of renal stone composition [20].

Joseph et al [2] suggested that stones with CT attenuation value of greater than 950 HU and stones required 7500 shockwaves failed to achieve fragmentation. Gupta et al [21] showed that the worst outcome of ESWL was in patients with calculus densities of more than 750 HU and diameters of more than 1.1 cm, and their clearance rate were only 60%. In our study, the success of ESWL treatment is almost always guaranteed when the CT attenuation value is less than 700 HU, while, at the same time, treatment failure is almost certain when the CT attenuation value exceeds 900. This is comparable to the results of recent studies [17,22,23]. Stone densities in the range of 700-900 HU may, or may not, respond successfully to ESWL treatment. Unlike Gupta et al [21], this study found that stone densities of more than 700 HU may fail to respond successfully to ESWL treatment. In addition, contrary to Gupta et al [21], this study revealed that stone diameters of up to 20 mm may still (depending on stone density) respond successfully to ESWL treatment. Contrary to Joseph et al [2], the results of this study clearly reveal that stones with densities exceeding 900 HU are difficult to fragment. However, unlike Joseph et al [2], up to

4600 shock waves may be attempted before seeking other types of treatment (i. e., percutaneous nephrolithotomy). Even though the results of this study have identified both stone density and size as significant contributors to ESWL treatment success rate, it also revealed that stone density is the determinant factor of treatment success for stone sizes of 20 mm or smaller.

To date, few clinical studies have compared the stone density with the outcome of ESWL *in vivo*. In a study of 65 patients, Joseph et al [(2)] showed that stones with densities less than 500 HU have 94% clearance rate and required a median of 2800 shockwaves, patients with stone densities of 500-1000 HU have 76% clearance rate and required a median of 3700 shockwaves, and patients with stone densities more than 1000 HU have 42% clearance rate and required a median of 7800 shockwaves.

Pareek et al [(24)] correlated calculus density with stone clearance in their study of 100 patients. They concluded that patients with residual calculi had a mean calculus density of more than 900 HU. However; Pareek et al [(24)] did not correlate the calculus density with fragmentation. The results of our study concurs with Pareek et al results in that stone clearance is unlikely when stone density exceeds 900 HU

The results of this study supports those of Joseph et al [(2)] in that stone density has an inverse relation with the ESWL success rate, and CT stone density has a positive correlation with the number of shockwaves needed for fragmentation. Also, the results of this study concurs with the results of previous studies [(25-27)] in that stone location has a significant effect on fragmentation success and clearance with lower calyceal stones have less success rates compared to other locations.

This study has some limitations including the limited number of the patients; therefore, larger number of patients is needed to achieve more significant results in the future studies. The other parameter was the study of stone chemical composition which can be predicted by measuring the density of urinary calculi using the dual–energy multidetector CT scan. This parameter was not assessed in our study because such types of CT scan are not yet widely available.

In this study, we recommend using non contrast CT (NCCT) scan as an initial diagnostic test to evaluate acute flank pain. Also it can be used to assess urinary stones prior to ESWL especially in patients with recurrent urinary stones as it is helpful to determine stone size and location and more importantly, stone density. This is valuable to choose the appropriate treatment option and to predict the success of ESWL to avoid unnecessary nonproductive ESWL.

In conclusion, ESWL treatment outcome is strongly, but inversely, dependent on stone density. Stones with CT densities of 700 HU or less undergo successful treatment requiring lesser number of shock waves and sessions, contrary to stones with CT densities more than 900 HU Large stones more than 2 cm and stones with lower calyceal location are resistant to ESWL.

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Cervicovaginal Smears' Classification Using the Bethesda System (TBS) 2001: A Cytopathological Study

Toqa J. Chkhaim[1] *MBChB MSc*, **Husam H. Ali**[2] *MSc FICMS*, **Liqaa R. Mosa**[3] *MBChB FICMS*, **Kifah H. Abdalghafour**[4] *DSP PhD*

[1]Pathologist in Ministry of health, Iraq, [2]Dept. of Pathology & Forensic Medicine, [3]Dept. of Gynecology & Obstetrics, College of Medicine, Al-Nahrain University, [4]Dept. of Pathology, College of Medicine, Baghdad University, Iraq

Abstract

Background The Bethesda System (TBS) aims to simplify cervical smear report and make it more reproducible and facilitates the communication between pathologist and clinician.

Objectives To evaluate 2001 Bethesda System of cervicovaginal smear classification in the diagnosis of different pathologies seen in women having different gynecological complaints.

Methods A prospective study of cervicovaginal smears that obtained from 360 female patients (aged 15-75 years) attending Gynecological Consultation Clinic in Al-Imamian Al-Kadhimiyian Medical City – Baghdad- Iraq for the period from November 2011 to April 2012. Smears were stained by Pap stain to evaluate according to Bethesda system 2001.

Results A total of 360 cases were evaluated, 317 cases (88%) had satisfactory smears for evaluation. 246 cases (68.3%) had negative cervical smears for intraepithelial neoplasia (TBS 2001). Seventy one cases (19.72%) had abnormal cervical smears (AS). Minimal cervical smear abnormalities (ASC-US, ASC-H, AGC, LSIL), includes (53) cases (74.64% of AS). HSIL (CIN- II, CIN-III, & carcinoma in situ), includes (18) cases (25.36% of AS).

Conclusion Pap smear is a screening test, it is not a diagnostic test; positive result indicates that there may be a problem and that further diagnostic procedures must be done. The Bethesda system is of validity in providing a uniform format for cervical cytology report.

Key words Pap smear, cervical intraepithelial Neoplasia (CIN), LSIL, HSIL, 2001 Bethesda System (TBS).

Introduction

The fundamental goal of cervical cancer screening is to prevent morbidity and mortality from cervical cancer. The optimal screening strategy should identify those cervical cancer precursors likely to progress to invasive cancers (maximizing the benefits of screening) [(1)].

Cytology (Pap test) screening has been very successful in lowering cancer incidence and mortality in countries where good quality screening is available [(2)].

According to the latest Iraqi Cancer Registry records (2008), cervical cancer ranks the 8th among the most common female cancers in IRAQ accounting for 0.8% of total female malignancies [(3)].

Fewer than 5% of women in developing countries have ever had a Papanicolaou (Pap) test; in contrast, 89% of women in the United States report having had a Pap test in the preceding 3 years [(4)].

High-income countries have effectively integrated Pap smear–based cervical cancer

screening services into both medical and public health services and have achieved reasonably high coverage rates, effectively reducing incidence and mortality over the past seven decades [5].

The expanding use of effective prophylactic vaccines for preventing infection with human papillomavirus (HPV) types 16 and 18, common etiologic agents for cervical cancer, offers even greater promise for eventual elimination of cervical cancer as a major public health problem [6].

The 20th century witnessed a remarkable decline in the mortality from cervical cancer in many developed countries; this achievement is directly attributable to the implementation of the Papanicolaou's (Pap) test [7]. In the 1930s, before Pap screening was introduced, cervical cancer was the most common cause of cancer deaths in women in the United States. Today, it is not even one of the top ten [8]. The Pap smear is a cytologic screening test used to detect cervical intraepithelial neoplasia (CIN) and early cervical cancer so that these conditions can be managed or treated to prevent disease progression to invasive cancer. Cervical cytology results are not diagnostic of CIN or cancer, as biopsy and histologic confirmation are required for diagnosis [9].

Terminology forms the basis for effective communication between the laboratory and clinician. The use of a uniform diagnostic terminology facilitates communication by establishing a common language that, in theory, does not vary significantly from cytologist to cytologist or laboratory to laboratory [10].

The Bethesda System 2001 and its 1991 and 2001 revision aim to simplify Pap smear reporting and make it more reproducible. It redefines the Pap smear request as a medical consultation [11].

The objective of this study is to evaluate 2001 Bethesda System of cervicovaginal smear classification in the diagnosis of different pathologies seen in women having different gynecological complaints.

Methods

The study is a prospective one. Cervicovaginal smears were obtained from 360 female patients with different gynecological complaints (aged 15-72 years) all were married and non pregnant attending Gynecological Consultant Clinic in Al-Imamian Al-Kadhimiyian Medical City, Baghdad, Iraq for the period from November 2011 to April 2012.In this study cervicovaginal smears were evaluated and assessed using, the Bethesda System (TBS) 2001 with special emphasis on premalignant lesions, with exclusion of cases which were unsatisfactory for evaluation. Patients were categorized according to the Bethesda System into:

- Cases of atypical squamous cells (ASC) including:atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells cannot exclude HSIL (ASC-H)
- Cases of low-grade squamous intraepithelial lesion (LSIL)
- Cases of high-grade squamous intraepithelial lesion (HSIL)
- Cases of atypical glandular cells (AGC)

Pap smear technique: Two cervicovaginal smears were prepared for each patient, after fixation with95% ethyl alcohol, slides stained by Pap stain [4,10].

Papanicolaou stain (progressive method):

1. **Rehydration:** put the fixed smear in 80% then 70% then 50% ethyl alcohol and then in tap water for each rinse 10 dips.
2. **Nuclear stain:** Harris Hematoxylin, put the smear in this dye for 45sec. to 1 minute**.**
3. **Rinse:** rinse the smear in 2 water rinses for each rinse 10 dips.
4. **Dehydration:** put the smear in 50%, 70%, 80% and 95% ethyl alcohol and for each rinse 10 dips.
5. **Cytoplasmic stain:** put the smear in Orange G-6 for 1¼ minute.
6. **Rinse:** rinse the smear in 3 rinses 95% ethyl alcohol and for each rinse 10 dips.
7. **Cytoplasmic stain:** Eosin Azur- 65 (EA65) for 3 minutes.
8. **Rinse:** rinse the smear in 3 rinses 95% ethyl alcohol and for each rinse 10 dips.

9. **Dehydration:** rinse the smear in 3 rinses absolute ethyl alcohol and for each rinse 10 dips.
10. **Clearing:** rinse the smear in 3 rinses xylene and for each rinse 10 dips.

Statistical analysis: Statistical analysis was done using student t-test. P value of less than 0.05 was considered statistically significant. The statistical significance of association between two categorical variables was assessed by chi-square test.

Results

The total number of pap smears was 360; 317 were adequate and 43 smears were inadequate for evaluation.

Clinical data of the total study sample:

Theage range was (15-75 years) with a mean age of (37.98 years ± 10.97).The chief complaints of the patients were vaginal discharge, postcoital bleeding, intermenstrual bleeding, postmenopausal bleeding, vaginal and perianal warts (Table 1).

Table 1. The classification of patients according to the clinical symptoms

Signs and symptoms	No.	%
Vaginal discharge	229	63.61
Postcoital bleeding	38	10.55
Intermenstrual bleeding	76	21.12
Postmenopausal bleeding	9	2.5
Vaginal & perianal warts	8	2.22
Total	360	100

Cytological cervical smear results of (360 cases) were categorized according to The Bethesda System (TBS) 2001 into the following [10]: 317 cases (88%) were satisfactory for evaluation (presence of endocervical/ transformation zone components with adequate squamous cellularity), 43 cases (12%) were unsatisfactory for evaluation (absence of endocervical / transformation zone components, autolysis, obscuring blood, obscuring inflammation and small amount of material). Two hundred and forty six cases (68.3%) had negative cervical smears for intraepithelial neoplasia (TBS 2001). Seventy one cases (19.72%) had abnormal cervical smears (AS), smears with intraepithelial lesions. In which:

a. Minimal cervical smear abnormalities. (ASC-US, ASC-H, AGC, LSIL) This category includes (53) cases (74.64% of AS: abnormal smears, 14.72% of studied group).

b. HSIL. (CIN- II, CIN-III, and carcinoma in situ). This category includes (18) cases (25.36% of AS, 5% of studied group).

LSIL, as a single entity, was the most common cytological abnormality 28 cases (39.43% of AS, 7.7% of studied group). ASC includes 14 cases (19.71% of AS, 3.88% of studied group); which is subdivided into: ASC-H includes 8 cases (11.26% of AS, 2.22% of studied group); ASC-US includes 6 cases (8.45% of AS, 1.6% of studied group).AGC includes 11 cases (15.5% of AS, 3.05% of studied group as demonstrated in table 2.

Table 2. The outlines of cytological examination of the Total study group

Cytology	No.	Group (%)	
		studied N = 360	AS N = 71
-ve cervical smear	246	68.3	
Inadequate	43	11.95	
ASC- US	6	1.7	8.45
ASC-H	8	2.2	11.26
LSIL	28	7.8	39.43
HSIL	18	5.0	25.36
AGC	11	3.05	15.5
Total	360	100	100

The mean age at the time of examination for patients with abnormal cervical smears was (39.91 ± 11.5 years). The mean age for patients with HSIL was (45.94 ± 12.3 years) which is higher than that for patients with minimal cervical smear abnormalities (36.88 ± 10.46 years). Also, the mean age for patients with LSIL (38.21 ± 14.3 years) was higher than that for patients with AGC (34.9 years ± 9.72) or ASC

(35.78 ± 9.79 years). The peak age interval for women with AGC was (30-39) years, for women with ASC was (30-39) years, for women with LSIL was (40-49) years (which was statistically significant), and for women with HSIL was (40-49) years (which was statistically not significant).

The frequency of clinical presentations for all patients considered as Abnormal Smear (AS) is as follow:

Vaginal discharge was the clinical presentation for (32) cases (45% of AS). Thirteen cases (18.3% AS) interpreted as LSIL, twelve cases (17% AS) interpreted as HSIL, three cases (4.2% AS) interpreted as ASC-H, two cases (2.8% AS) as ASC-US, and two cases (2.8% AS) as AGC.

Recurrent cervicitis was the clinical presentation for (20) cases (28.15% of AS). Of which, nine cases (12.7% AS) interpreted as LSIL, six cases (8.5% AS) as HSIL, two cases (2.8% AS) as AGC, two cases (2.8% AS) as ASC-US, and one (1.4% AS) as ASC-H.

Intermenstrual bleeding was the clinical presentation for (17) cases (24% of AS). Of which, seven cases (9.8% AS) interpreted as LSIL, four cases (5.6% AS) interpreted as AGC, three cases (4.2% AS) as HSIL, two cases (2.8% AS) as ASC-H, and one (1.4% AS) as ASC-US.

Post coital bleeding was the clinical presentation for (16) cases (22.55% of AS). Of which, five cases (7% AS) interpreted as LSIL, four cases (5.6% AS) interpreted as AGC, three cases (4.2% AS) as ASC-US, two cases (2.8% AS) as ASC-H, and two cases (2.8% AS) as HSIL.

Vaginal and perianal warts were the clinical presentation for (4) cases (5.6% of AS). Of which, two cases (2.8% AS) interpreted as LSIL, one (1.4% AS) as AGC, and one (1.4% AS) as ASC-H.

Post-menopausal bleeding was the clinical presentation for (2) cases (2.8% of AS), of which one case (1.4% AS) interpreted as LSIL, and the other one (1.4% AS) as HSIL (Table3).

Table 3.The frequency and percent of clinical presentation for AS patients

Clinical features	ASC-US		ASC-H		AGC		LSIL		HSIL		Total		P value
	N	%	N	%	N	%	N	%	N	%	N	%	
Vaginal discharge	2	2.8	3	4.2	2	2.8	13	18.3	12	17	32	45.1	0.129
Intermenstrual bleeding	1	1.4	2	2.8	4	5.6	7	9.8	3	4.2	17	24	0.799
Postcoital bleeding	3	4.2	2	2.8	4	5.6	5	7	2	2.8	16	22.5	0.238
Vaginal & perianal warts	0	0.0	1	1.4	1	1.4	2	2.8	0	0.0	4	5.6	0.643
Postmenopausal bleeding	0	0.0	0	0.0	0	0.0	1	1.4	1	1.4	2	2.8	0.865
Total	6	8.5	8	11.2	11	15.5	28	39.3	18	25.5	71	100	
P value	0.542		0.886		0.322		0.942		0.170				0.668

Discussion

In the present study, the results of cytological examination and their interpretation are classified according to The Bethesda System 2001 (TBS) for reporting the results of cervical cytology which is developed as a uniform system of terminology that would provide clear guidance for clinical management. The current study is the 2nd one in Iraq that uses The Bethesda System 2001 (TBS) in the evaluation and interpretation of cervicovaginal smears. However, the first study was done by Al-Guraity (2006) [12] which was retrospective study and including 91 cervicovaginal smears; while present study is a prospective one and the sample size is 360 cervicovaginal smears evaluated by The Bethesda System 2001 (TBS).

Minimal cytologic abnormalities are more common than HSIL. Al-Guraity (2006) [12] reported the same observation (according to TBS 2001). Al-Ani (2001) [13], Al-Ruba'ee (2002) [14], Apgar and Brotzman (1999) [15] reported the same observations (according to TBS 1991). It includes:

LSIL as a single entity, was the most common cytological abnormality in the present study and it includes CIN I and koilocytic atypia. It represented about 61% of SIL (squamous

intraepithelial lesions) findings in cytology. According to similar study in our country; this was lower than that reported by Al-Guraity (2006) which was (88%) [12], because this study is prospective and with a larger sample size, however they are lower than Margolis et al (1999) and other studies due to lower frequency of HPV in the eastern population [16,17], due to widespread difference in the prevalence of risk factors, different sexual habits and probably the availability of screening programs [18,19].

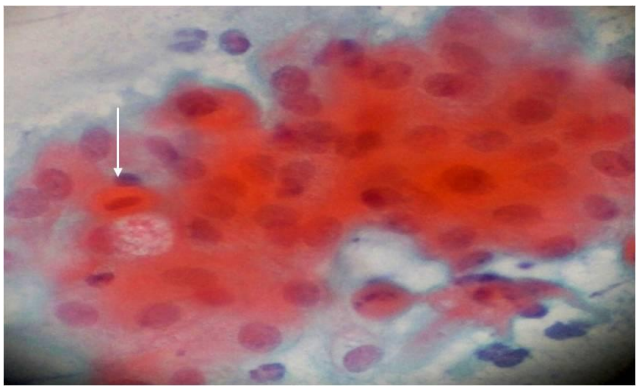

Fig. 1. Cervical smearLSIL: nuclear enlargement with pyknosis and cytoplasmic orangophilia (arrow) X 40 (Pap stain)

ASC was the 2nd common minimal cytological abnormality in this study and it is lower than similar study in Iraq Al- Guraity (2006) [12], because this study is prospective and with a larger sample size.

AGC represented (15.49% AS), this was higher than that reported by Al-Guraity (2006) which was (3.9% AS) Al- Guraity (2006) [12], Al-Rubai'ee (2002) which was (9% AS) - according to TBS 1991[14], also more than that reported by Fadwa (2001) which was (5.7% AS) - according to TBS 1991 [18]. Also our results were higher than Burja et al (1999), who found that incidence of AGUS in their studies were (2.1% Total studied Group) [20].

So different studies gave different rates and number of cases included in different studies may play a rule in the discrepancy between rates. AGC is relatively uncommon cytological interpretation, occurring in approximately 0.18 to 0.74% of cervical smears in screening programs, and representing about 4% of the abnormal cytological findings [21], which is less than our results.

Modifications were incorporated into the 1991 Bethesda System that streamlined the terminology and clarify controversial and borderline cytological abnormalities that lead to introduction of TBS 2001 [10].

In this study HSIL represented (25.36% AS) which is much more than that of Al-Guraity (2006) [12] and other studies in the nearby countries using TBS 1991 for classification Fadwa (2001) [18]. Also, it is much more than that reported by Al-Rubai'ee (2002) using TBS 1991 for classification [14]. Lower percentage was reported by Wertlake (1999), who reported HSIL in (8.5% of AS) [22]. Present study which using TBS 2001 and is prospective taking large size of samples. Current study's results are somehow nearly similar to western studies; unfortunately in these years we have highly increase in STDs (sexually transmitted diseases), and also probably due to the unavailability of screening programs for cervical cancer in Iraq.

ASC/ LSIL ratio was 0.5 in the present study which is lower than that reported by Al-Guraity (2006) [12], which was (1.09). Al-Rubai'ee (2002) reported ASCUS/LGSIL ratio was (1.1) [14], (2.1) reported by Al- Ani (2001) [13] and Davey et al (2000) reported ASCUS/LGSIL ratio was (2.0) [19], with about 80% of laboratories reporting ratios between (0.64) and (4.23) [19].

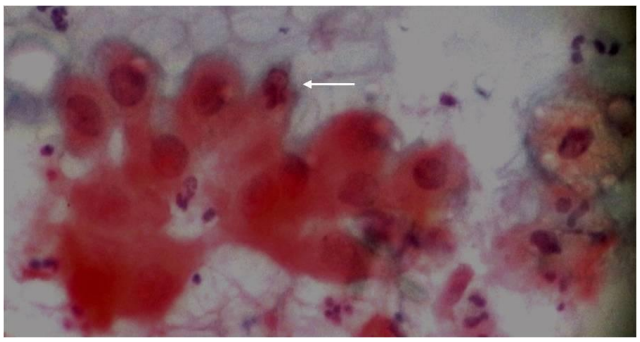

Fig. 2. Cervical smear shows ASC-H: nuclear enlargement with mild hyperchromasia (arrow) X 40 (Pap stain)

LSIL/ HSIL ratio was 1.6 in the present study which is much lower than that reported by Al-Guraity (2006) 7.3 [12]. Al-Rubai'ee (2002) reported LGSIL/HGSIL was 9.1 [14], and Al-Ani (2001) reported LGSIL/HGSIL was 7.0 [13]. The ratio in this study was slightly lower than that reported by Al-Alwan (2001) [23] which were 2.3; and Wertlake (1999) [22] reported a ratio of 3.

As previously mentioned, minimal cytological abnormalities are more common than HSIL in the present study and this also reflects the difference in the incidence of cervical cancer in our country compared to western countries that could be attributed to the promiscuity at early age and multiple sexual relations. In Islamic countries the circumcision, strict observance of religion and, presence of principles and laws that prevents the illegal relationships and extramarital relations may explain the lower incidence of cervical cancer in Iraq compared to western countries [24, 25].

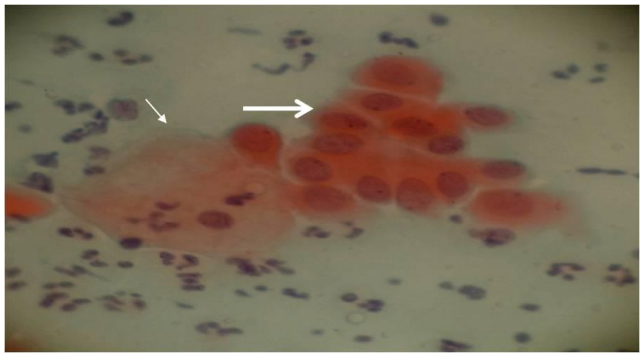

Fig. 3. Cervical smear shows HSIL:increased N/C ratio, irregular nuclear membrane and hyperchromasia (wide arrow) X40 (Pap stain); narrow arrow pointed at superficial squamous cell.

Age has been correlated with an increasing incidence of malignancies, and there is also an age correlation with the severity of the disease in precancerous lesions [23]. In present study, the mean age for patients with abnormal cervical smears was 39.91 years, the mean age for patients with LSIL was 38.21 years, and the mean age for patients with HSIL was 45.94 years. The risk of having LSIL was higher in women aged 40 years and more, as well as women with HSIL (the peak age interval for women with LSIL was 40-49 years which was statistically significant and that for HSIL was 40-49 years which was statistically not significant.

Al-Alwan (1995) reported a peak frequency of mild dysplasia in the age group 30-39 years [16], Al-Ani (2001), Al-Ruba'ee (2002) and Ronald et al reported that women aged 40 years and more are at higher risk of harboring SIL especially the higher grade lesions [13, 14, 26]. Al-Guraity (2006) reported peak frequency of LSIL to be in the (40-49 years) interval, and peak frequency of HSIL was between (50-59 years) [12]. Others, like Blomeur et al (1999) reported a mean age of 35 years to be more likely to have SIL and also Al-Badri (2000) reported the mean age of 39 years respectively [25, 27].

The results of current study, comes in concordance with that of other Iraqi and western studies. Other studies in UK reported that the mean age specific rate for SIL occurs in late 20s [28]. The wide differences, in the mean age of SIL could be explained by the widespread difference in the prevalence of risk factors, different sexual habits, design of study, the availability of screening programs and sample size [12].

The most common complaint that was recorded in the present study and by other studies in Iraq like that of Al-Ruba'ee (2002) [14], Al-Guraity (2006) [12]; was vaginal discharge, followed by intermenstrual bleeding, postcoital bleeding, postmenopausal bleeding, and vaginal and perianal warts. There was no statistically difference found in the incidence of abnormal cervical smears between patients regarding these different clinical features. The incidence of intraepithelial lesions has no significant relation with vaginal discharge or intermenstrual bleeding; (there was a statistically significant relation between vaginal discharge and ASC-H, ($P< 0.05$) which is, similar to results of previous studies from Iraq [12-14], (no statistically significant differences were found in the incidence of SIL of any grade with the above clinical features).

Regarding SIL, many literatures reported that CIN (SIL) is usually free from symptoms and that

the condition owes its existence as an entity only to assign. Al-Alwan (1987) reported that, the coexistence of CIN with abnormal vaginal bleeding is mostly due to the associated cervical lesions or other systemic abnormalities in these patients [(29)].

About 20% of cases interpreted as SIL had Intermenstrual bleeding (IMB). Results of the present study agrees with that of Al-Alwan (1987) [(29)] but disagrees with that of Al-Guraity (2006) [(12)] and Al-Anbari (2002) [(24)]; and this may be due to other causes that lead to spotting, irregular menstrual bleeding as hormonal imbalance, or other cause may be due to chronic or severe cervicitis. Postmenopausal bleeding was found in about 4.3% of postmenopausal women who had SIL, which was much lower than that reported by Al-Guraity (2006) [(12)], which could be attributed to different sample size and being a prospective study in comparison to that of Al-Guraity (2006) [(12)] which was retrospective.

Postcoital bleeding was found in about 18% of patients with SIL in current study which is higher than that reported by Al-Guraity (2006) [(12)], but there was agreement with that reported by; Al-Alwan (1987) [(29)], Al-Anbari (2002) [(24)], and Rosenthal et al (2001) [(30)].

Also, Rosenthal et al (2001) reported that although, invasive cancer in women with PCB varies in literature from 0% to 5.4%; in most of the studies it was more frequent than general population. PCB was associated with CIN in 5%-32.7% of cases in different studies [(30)].

A normal cervical smear in women with PCB does not rule out the possibility of SIL or invasive cancer, but most women with postcoital bleeding will have no serious abnormality [(26)].

William (2002) [(31)] believes that, the Pap test is a screening test for malignant and premalignant changes of the cervix. A positive result indicates that there may be a problem and that further, diagnostic procedures (colposcopy or biopsy) must be done. The Pap test is not diagnostic test; it cannot be used to exclude a cancer of the cervix for a person who has symptoms that could be due to a cervical cancer.

This is the single most important lesson to learn: if you have a symptom or a finding that could be due to a cancer of the cervix; a normal Pap test never excludes the possibility of cancer [(31)].

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Correspondence to Dr. Toqa J. Chkhaim
E-mail: toqa79@yahoo.com
Mobile: + 964 7816874299
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Anatomical Variations of Extrahepatic Biliary System

Lutfi G. Awazli *DGS, HDLM, FICMS*

Laser Institute for Postgraduate Studies, University of Baghdad, Baghdad, Iraq

Abstract

Background Variations in the anatomy of gallbladder, bile ducts and the arteries that supply them are important to the surgeon during cholecystectomy, because failure to recognize them may lead to inadvertent iatrogenic injuries.

Objective To evaluate the type and frequency of anatomical variations of extra hepatic biliary system encountered during cholecystectomy.

Methods One hundred and fifty patients with gallstones underwent cholecystectomy at Baghdad Teaching Hospital. There comprised 112 females and 38 males with age range between 20-80 years. Open (33 cases) and laparoscopic (117 cases) cholecystectomies were done. Extra hepatic biliary tree was carefully dissected to study the variations in the anatomy of the gallbladder, bile ducts, and the arteries that supply them.

Results There were only three important vascular and four important ductal anomalies while gallbladder anomalies were rare. The total numbers of the extrahepatic biliary anomalies were 81 cases (incidence 54%), and included vascular anomalies (60 cases = 40%); ductal anomalies (18 cases = 12%); gallbladder anomalies (3 cases = 2%); mostly occurred as Phrygian cap (2 cases = 1.3%). The higher incidence of anatomical abnormalities was found in females 80% (65 cases) while in males 20% (16 cases).

Conclusion Anomalies of the vascular and ductal components of the extra hepatic biliary tree are relatively common (the former occurring much more frequently than the latter). Failure to recognize them during biliary surgery leads to iatrogenic injuries and can increase morbidity and mortality.

Key Words Extra hepatic biliary tract; biliary anomalies, cholecystectomy.

Introduction

The biliary tract is the site of great variation and even gross anomalies and their clinical significance is variable [1,2]. These anomalies can represent a major challenge especially to unprepared and unaware surgeons for failure to recognize them at operation may lead to disaster [3,4].

The anatomy of the biliary system has been the subject of extended research for many years largely because of their surgical importance in cholecystectomy, and interest has been centered on the extrahepatic biliary tree because it is frequently abnormal [5]. Many studies have attempted to determine a standard length, diameter, and thickness of various portions of the ductal system but significant normal variability in duct size and length may be encountered [6].

There is a wide difference of opinion which still exists regarding basic detail of extrahepatic biliary anatomy, and it is pertinent at this point

to consider why these discrepancies occur, there are probably a number of reasons [5]:

1. It is not possible to compare the results derived from different methods (e.g. radiological and dissection) [5].
2. One cannot necessarily expect, using the same investigative technique to produce identical measurements and observation on different basic materials (i.e., cadaver and operative specimens) [5].
3. The operative field allows less scope for the dissection of the anatomical details which are best demonstrated on resin-cast material [5].
4. Various radiological techniques, i.e., intravenous cholangiography, per-operative cholangiography, post-operative T-tube cholangiography, ERCP (Endoscopic retrograde cholangio-pancreatograghy), PTC (Percutaneous transhepatic cholangiograghy), do not necessarily produces exactly the same measurable results in the same patient [4].

Many studies reported that the incidence of biliary anomalies varies from 15 to 66 percent [1,3,5,7-11]. This study aims at describing some anatomical variations of the extra hepatic biliary system that face the surgeon during cholecystectomy and determine the type and frequency of each anomaly.

Methods

This is an observational study of one hundred and fifty consecutive patients with calculi of the biliary system operated on as elective cholecystectomies, all of them done in Baghdad Teaching Hospital for a period of one year (from 1st October 1999 to 1st October 2000). There were 112 females and 38 males, with age range of 20-80 years and a mean age of 46 years. In general, the clinical presentation of patients was attacks of upper abdominal pain, vomiting, with or without jaundice. Preoperative investigations included abdominal ultrasound and liver function tests, which indicate the presence of gallstones or bile duct stones. Operative technique included laparoscopic method (117 cases = 78%) while conventional open method (33 cases = 22%) with or without common bile duct (CBD) exploration, 24 cases (16%) by right subcostal, and 9 cases (6%) by right paramedian incision.

At the time of operation, a detailed sketch was made by the surgeon, by elevation of the anterior margin of the right lobe of the liver with retraction of the stomach, duodenum, and colon to expose the gallbladder (GB), then by careful blunt dissection of the hepatoduodenal ligament and the Calot's triangle which is necessary in order to identify the structures in or around this region and avoid any accidental injury to the extrahepatic biliary ducts and blood vessels, and also to show the main anatomical features, and in particular the relations of the common hepatic, common bile, and cystic ducts, and the course and relations of the right hepatic and cystic arteries, and also to determine the type and frequency of each anomaly and its surgical significance.

Results

The total series of 150 cholecystectomies have been done in this study, included 112 females (74.7%) and 38 males (25.3%) with a peak incidence in the fifth decade of life and a mean age of 46 years, as shown in (Table 1).

Table 1. Age and Sex distribution

Age (Yr.)	20-29	30-39	40-49	50-59	60-69	70-80	Total (%)
Male	2	5	12	8	6	5	38 (25.3)
Female	7	22	36	23	18	6	112 (74.7)
Total	9	27	48	31	24	11	150 (100)

*Female: Male ratio = (112/38) = 3:1

The total number of extrahepatic biliary anomalies were 81 out of 150 cases (incidence 54%), and these divided into vascular (40%), ductal (12%), and GB anomalies (2%), (Table2).

Vascular anomalies

The incidence of vascular anomaly is high (40%), The commonest was the accessory cystic artery (18%), though not much less common is the anterior transposition of the cystic artery, or the right hepatic artery (16%), while the incidence of the caterpillar hump right hepatic artery was much less (6%).

Ductal anomalies

The incidence of ductal anomalies was much less than that of arterial anomalies (18 cases = 12%), The commonest is a long cystic duct with or without low fusion with common hepatic duct (CHD) occurring in 8 cases (5.3%), while other ductal anomalies like short cystic duct, high fusion of cystic duct with CHD or right hepatic duct (RHD), and accessory hepatic ducts were found in (3 cases = 2%), (3 cases = 2%), and (4 cases = 2.7%) of patients respectively.

Regarding the accessory hepatic ducts, all of them arose from the right lobe of the liver and drained either into the neck of GB (one case) or the CHD (3 cases). The length and diameter of these ducts were extremely variable.

Gallbladder anomalies

In this study, there were only three cases (2%), and these included Phrygian cap (2 cases), and the other interesting case which is not reported in textbooks or other studies, in this case the GB fundus passed through the liver substances from the inferior (visceral) surface to protrude out at the anterior surface, making a hole in the liver (Table 2).

Table 2. Extrahepatic biliary anomalies in cholecystectomies and its several subtypes

Anomalies		No. (%)	Total
Vascular anomalies	Accessory cystic artery Anterior cystic artery or anterior right hepatic artery Caterpillar hump right hepatic artery	27 (18) 24 (16) 9 (6)	60 (40)
Ductal anomalies	Long cystic duct Short cystic duct High fusion of cystic duct with common hepatic duct Accessory hepatic ducts	8 (5.3) 3 (2) 3 (2) 4 (2.7)	18 (12)
Gallbladder anomalies	Phrygian cap Gall bladder fundus pass through the liver	2 (1.3) 1 (0.7)	3 (2)
Total		81 (54)	

Associated anomalies

The number of patients in whom vascular, ductal, and GB anomalies actually coexist is small, only 5 cases (3.3%).

Relation of the cystic artery with the Calot's triangle

The cystic artery was found inside the Calot's triangle in 144 cases (96%), while in 6 cases (4%) outside and in these cases they were found inferior to the cystic duct.

Operative technique

Included laparoscopic cholecystectomy were successfully done in 117 cases out of the total 150 cases of cholecystectomy (78%), while the rest 33 cases (22%) done by conventional open cholecystectomy.

The number of the anomalies recognized by laparoscopic cholecystectomy was 64 out of total 81 anomalies, while other 17 cases recognized by conventional open cholecystectomy.

Only 4 cases of laparoscopic cholecystectomy converted to open method due to extensive adhesions with unclear anatomy (3 cases), and uncontrolled bleeding (1 case), and this gives a conversion rate (3.3%).

Table 3. Comparison between this study and other studies regarding vascular anomalies

Studies	Anterior cystic or anterior RHA (%)	Accessory cystic artery (%)
Our study (2000)	16	18
Khamiso (2010) [8]	2.67	1
Gupta (2003) [15]	-	15
Bhanasali (2003) [16]	-	20
Adkins (2000) [3]	-	12
Shwartz (1999) [13]	15	25
Stremple (1986) [14]	20	25
Benson (1976) [5]	20.7	26.4
Moosman (1951) [17]	19.6	25.2

Discussion

Many studies reported that the incidence of biliary anomalies varies from 15 to 66 percent. [1,3,5,7-11] In this study the incidence of anatomical abnormality in the disposition and relations of the extrahepatic bile ducts and arteries is (54%), so it is within the range reported by others, thus the surgeon will meet some anomaly in every other case upon which he operates. This in keeping with the statement made by Hand (1973) [6] "It is difficult to know what is normal and what is abnormal". Although the incidence of anomalies is high, there are in fact a relatively few surgical important ones (three vascular and four ductal) and all these were readily recognized at operation.

Vascular anomalies

Vascular anomalies (40%) were more common than ductal anomalies (12%). Commonly the cystic artery passes superior and medial to the cystic duct within the Calot's triangle [3] as in this study (96%), while it is found outside in 6 cases only (4%), inferior to cystic duct especially when there is high insertion of this duct. So it is important to be aware of the situation when no artery is seen in Calot's triangle, because various abnormalities in position may exist and overlooking them result in sever hemorrhage [12].

The commonest vascular anomalies are:

I. Accessory cystic artery (18%):

This high incidence was also reported in many studies (no statistical significant difference between our study and other studies: $P > 0.05$), as shown in (Table 2) [3,13-17]. Therefore, after carefully ligating or clipping one artery, the surgeon must search carefully for the possibility of another supply which may have any source of origin, and if not identified this may be torn and bleeding may obscure the operative field and hurried blind clamping may produce a disaster [14].

II. Anterior transposition of the cystic artery or the right hepatic artery (RHA) anterior to the CHD or CBD:

This anomaly was found in (16%), which was also reported by other studies as shown in (Table 3), again there is no statistical significant difference between our study and other studies: $P > 0.05$. It is clinically important to note especially when doing an exploration of CBD, and when the anterior cystic artery being ligated there is always a possible risk of direct injury to either CBD or CHD, depending on where the anterior cystic artery runs, how closely it is related to the ductal structure and how far proximally the ligation is placed [14].

III. Caterpillar hump right hepatic artery: The incidence of this variation was 6% in this study. It is within the range reported by other studies (1-12.9%) [8,17-20]. In this case the right hepatic artery replaces the cystic artery within the Calot's triangle, and it is tortuous and projects forwards to the right of the CHD. It is a dangerous anomaly because it may be mistaken for the cystic artery so ligation can lead to fatal complication in the presence of impaired liver functions [21, 22].

Ductal anomalies

The incidence of significant anomalies of the extrahepatic bile ducts ranges from 10 to 28 percent in autopsy series [23-27]. The cystic duct varies in length as well as in the level and pattern of conjunction with the common hepatic duct [23].

The commonest ductal anomalies are:

1. Long cystic duct with abnormal low fusion with the CHD. In this study it was found in (5.3%) of cases. Under this circumstance the cystic duct is invariably longer than normal. It runs alongside and parallel with the CHD, before joining it. In this case a variable length of the cystic duct is tightly bound to the CHD before they actually fuse [16]. Thus, extensive dissection of the distal portion of the cystic duct can produce devascularization of a segment of CBD, subsequently ischemia, fibrosis and stricture at the level of junction of cystic duct with the CHD [10].

2. Short cystic duct: This anomaly was found in 3 cases (2%). In this condition, the cystic duct is very short (less than 0.5 cm in length). The main danger of this anomaly when the surgeon try to visualize the cystic duct by vigorous traction on the GB, so producing marked angulation and tenting of the CHD or CBD which may then be caught in a clamp or clip [3].

3. High fusion of the cystic duct with CHD or RHD: It was found in 3 cases (2%). In this condition the cystic duct enters the confluence of the right and left hepatic ducts making trifurcation, so the right or left hepatic ducts may be damaged during cystic duct ligation or clipping, furthermore any tenting produced by traction could compromise the lumen at the confluence if a tie was applied [3].

4. Accessory hepatic (bile) ducts: It is the most interesting abnormality of the ducts, because there is a wide variation in its incidence between literatures and quoted as varying from 0.67 to 31 percent (as shown in table 4) [8,13,17,27-29], in addition that he risk of injury to an accessory duct without knowledge that it has been torn or avulsed is present in every case of cholecystectomy [28], because they are infrequently seen and difficult to recognized due to their unusual position and commonly so narrow in caliber in addition that bile flow during anesthesia is commonly decreased [25], or acute and chronic cholecystitis produces enough inflammatory changes which obscure the ductal structures. The incidence of accessory bile ducts in our study is less than that reported in literatures, (the difference is statistically significant: $P < 0.05$), as shown in table 4. The explanation is that, the high incidence occurs in studies who dissect resin- casts in cadaver [17,27,29] with more meticulous dissection technique might be responsible for the result. Other explanation is that, some surgeons might not be aware of the possibility of the presence of accessory bile ducts and certainly is not in the habit of looking for them at operation, and also the in availability of pre- and peroperative cholangiogram in this study.

Table 4. Comparison between this study and other studies regarding accessory hepatic ducts

Studies	Accessory hepatic ducts (%)
Our study (2000)	2.7
Khamiso AH(2010) [8]	0.67
Shwartz (1999) [13]	15
Lichtenstein and Nicosia (1970) [28]	10
Healey and Schroy (1953) [27]	28
Johnston and Anson (1952) [29]	31
Moosman (1951) [17]	16

Biliary tract injury

These injuries are frequently related to surgical inexperience and biliary tract anatomical variations which may be difficult to identify during laparoscopic surgery. Moossa et al. (1992) [30], emphasized that the presence of bile duct aberrations does not excuse bile duct injury and that intraoperative diagnosis of anatomical variations of the biliary tract contributes greatly to the safety of cholecystectomy. Many of the biliary injuries following cholecystectomy are not recorded in the reports, so it is difficult to know their true incidence [30-33]. In many studies it was found that injuries to the CBD have been reported in up to 0.5 percent (usually 0.2- 0.3%) of patient underwent open chole-cystectomy [34,35], while in laparoscopic cholecystectomy the initial studies was approximately 1% [36,37], but recently, the overall incidence of laparoscopic bile duct injury was 0.6% (range 0.1-2.9%) [38]. In this study, laparoscopic cholecystectomy has been associated with bile duct injuries in two cases (1.7%) while none in conventional open method and these injuries are acceptable because they are within the range reported by other studies as mentioned in the previous paragraph.

In conclusion, anomalies of the vascular and ductal components of the extrahepatic biliary tree are common; the former occurring much more frequently than the latter. Inexperience of the surgeon with the anatomical variations and the in availability of the pre- and per- operative cholangiogram were noted as common factors in most of iatrogenic biliary injuries during cholecystectomy. Laparoscopic cholecystectomy was performed in the majority of this patients with acceptable rate of injury (1.7%) because it is within the range reported by other studies (0.1- 2.9%). Open cholecystectomy continues to be a safe and effective means of treating anatomical variations of the extra hepatic biliary tree.

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E-mail: lutfigh@yahoo.com
Mobile: + 964 7701500758
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A Molecular and Comparative Study of Type-able and Non-type-able *Haemophilus influenzae* isolated from different clinical samples in Hilla, Iraq.

Bara' H. Hadi *MSc*, **Mohammed S. Abdul-Razzaq** *PhD*

Dept. of Microbiology, College of Medicine, Babylon University, Iraq

Abstract

Background *H. influenzae* bacteria classified as type-able and non-type-able according to the presence or absence of capsule which is correlated with site of isolation.

Objectives To isolate *H. influenzae* from different clinical samples and differentiate both capsulated (type-able) and non-capsulated (non-type-able) one by molecular detection method and to make a comparison between the two types by cultural, molecular, and clinical aspects.

Methods A total of 220 clinical samples were aseptically taken from throat, ear, eye, sputum and CSF of patients attended three main hospitals in Hilla city, Iraq during the period from Feb. 2012 to Jun. 2012. All samples were subjected to bacterial cultivation, standard bacteriological method and molecular detection method. Other primers were used to detect the presence or absence of capsule using Bex A, Bex B, while p2 primer was used to detect non-type-able. Among capsulated one a specific primers (Hib, bex) targeting *H. influenzae* type b were used.

Results 29 (13.2%) out of 220 clinical samples give presumptive detection and isolation of *H. influenzae*, of these only 10 (34.5%) were positive using X (haemin) and V (Nad) tests. Using PCR, only 6 out of 10 (60%) were positive, out of 6 isolates only 2 (33.3%) were capsulated (type-able) and 4(66.6%) non-type-able, out of 2 capsulated 1 (50%) was type b *H. influenzae*.

Conclusion Specific genetic marker should be used to detect both types. Many non-type-able *H. influenzae* isolates are also important cause of upper respiratory tract diseases including pharangitis, otitis media, and conjunctivitis. Using serotype specific gene targeting type b (Hib) is important among patient with meningitis and lastly separation between type-able and non-type-able according to the site of isolation.

Keyword *H. influenzae* , genetic marker, serotype specific gene, Hib, Bex(A, B) P6, NTHI, X,V factors

Introduction

Haemophilus influenzae (*H. influenzae*) is a Gram negative coccobacillus whose environmental niche is primarily restricted to the human respiratory tract. The genus name reflects its absolute requirement for heme and the species name reflects the erroneous initial belief that this organism was the causative agent of influenzae [(1)]. Generally, it is difficult and time-consuming to detect *H. influenzae* by using traditional method. So, PCR-based method targeting the outer membrane protein (OMP) represents specific diagnostic targets. OMP P6 is highly conserved among most strains of *H. influenzae* [(2)]. It is a peptidoglycan-associated lipoprotein constitutes 1-5% of all OMPs. Unlike P2 protein, P6 protein shown a very high homology (97%) in the amino acid

analyses of type b and non-type-able *H.influenzae,* which shows that this protein is stable and conserved [3]. Other OMP is P2 which is a major protein comprises more than 50% of the OMPs. This protein exists on the outer membrane as a trimer and act as a prion, so, it is mostly located on the outer membrane of non-type-able *H. influenzae* [4].

H. influenzae is also variable for the presence of polysaccharide capsule and is classified on the basis of production of polysaccharide capsule, strain types a through f produce antigenically distinct capsules and non-type-able strains produce no capsule [5]. Others classify *H. influenzae* into 3 main categories: nonencapsulated strains, encapsulated type b strains, and capsulated non-type b strains (types a and c-f) where type b is the most virulent form [6]. Several studies have demonstrated molecular capsule typing methods to be more sensitive and specific than other methods [7].

Encapsulated *H. influenzae* isolates contain genes for the production of their respective polysaccharides capsules at the cap locus which is composed of three distinct regions, designated region I to III. The genes contained within regions I and III, designated bex DCBA and hcs AB, respectively, are highly conserved across all six capsular types and are required for transport of capsule constituents across the outer membrane [8,9]. Yet, region II gene encodes capsule type a-through f-specific proteins and thus varies by serotype.

The organization and genetics of the cap locus are complex, duplications, partial loss, and complete loss of the cap locus can occur and give different results [10]. Early studies revealed that all isolates of *H. influenzae* are different in terms of pathogenic potential. It is very important to mention that most systemic isolates express the type b capsule, whereas most respiratory tract isolates contain unencapsulated, referred to as non-type-able [11]. These are more commonly part of the normal flora, less invasive and frequently involved in opportunistic respiratory tract infections. Many classification approaches showed that most non-type-able *H. influenzae* (NTH1) were genetically quite distinct from type b (Hib) strains and more heterogenous, yet, ribotyping and ERIC (Enterobacterial Repetitive Intergenic Consensus), PCR have been used to relate strains. The horizontal exchange of *H. influenzae* genetic loci between strains due to natural DNA transformation make the classification complicated and may explain the differences seen using different methods [12].

The objectives of this study was to isolate *H. influenzae* from different clinical samples and differentiate both capsulated (type-able) and non-capsulated (non-type-able) one by molecular detection method and to make a comparison between the two types by cultural, molecular, and clinical aspects.

Methods

Samples and Bacterial culture

A total of 220 clinical samples were taken from different clinical samples from patients attending the three main hospitals (Babylon Hospital for Maternal and Pediatrics, Al-Hilla Surgical Teaching Hospital and Merjan Medical City) during the period from February 2012-June 2012. The samples were transported using specific transport media and processed on blood, chocolate agar and tryptic Soya agar sublimated with X, V disc and subjected to standard bacteriological method and incubated in 5% CO_2 at 37^{o}C for 24 hrs., biochemical tests like catalase, oxidase, urease, indole, nitrate reduction, carbohydrate fermentation was done according to MacFaddin [13].

DNA extraction from Gram-negative bacteria

This method was performed according to the genomic DNA purification kit supplemented by a manufacturing company (Promega, USA).

Molecular method used in detection of type-able and non-type-able *H.influenzae*

H. influenzae was detected by PCR, using 20 µl PCR reaction mixture as in table 1. Primer used and thermal cycle condition were illustrated in table 2. The amplification product was separated on (1-1.5%) agarose gel containing ethidium bromide for 45 min. at 70 V. The size of the

amplicons was determined by comparison to the 100 bp allelic ladder (Promega, USA).

Table 1. Contents of reaction mixture

No.	Contents of reaction mixture	Volume (µl)
1	Green master mix	5
2	Upstream primer	3
3	Downstream primer	3
4	DNA template	5
5	Nuclease free-water	4
Total volume		20

Table 2. Primers sequences and thermal cycler conditions

Genes	Primer sequence (5'-3')	Size of product bp	PCR condition
P6F **P6R**	5-AACTTTTGGCGGTTACTCTG-3 5-CTAACACTGCACGACGGTTT-3	351	95 °C 10 min 1X 95 °C 30 sec 55 °C 1 min 30X 72 °C 2 min 72 °C 5 min 1X 95 °C 10 min 1X [31]
P2F **P2R**	5-GTTCACGTTTCCACATTAAAGC-3 5-CACGACCAAGTTTTACTTCAC-3	186	95 °C 30 sec 55 °C 1 min 35X 72 °C 2 min 72 °C 5 min [32]
Bex A F **Bex A R**	5-CGTTTGTATGATGTTGATCCAGAC-3 5-TGTCCATGTCTTCAAAATGATG-3	343	95 °C 2 min 1X 95 °C 30 sec 54 °C 30 sec 30X 72 °C 45 sec 72 °C 5 min 1X [33]
Bex B F **Bex B R**	5-GGTGATTAACGCGTTGCTTATGCG 5-TTGTGCCTGTGCTGGAAGGTTATG	567	95 °C 2 min 1X 95 °C 30 sec 54 °C 30 sec 30X 72 °C 45 sec 72 °C 5 min 1X [21]
Hib F **Hib R**	5-CCTCGCAATGCAGTTTATGGTCC-3 5-AAGCGGGAATTTGATACCTGATGC-3	774	94 °C 3 min 1X 94 °C 30 sec 60 °C 1 min 37X 72 °C 40 sec 72 °C 5 min 72 °C 1 min [34]
Bex F **Bex R**	5-TATCACACAAATAGCGGTTGG-3 GGCCAAGAGATACTCATAGAACGTT-3	81	95 °C 5 min 1X 95 °C 25 sec 57 °C 40 sec 35X 72 °C 10 min 1X [35]

Results

Among the 220 clinical samples only 29 (13.2%) isolates gave presumptive detection of *H. influenzae* and out of these 29 isolates only 10 were positive by using X (haemin) and V (NAD) tests that are required for growth of *H. influenzae* and these isolates were distributed and isolated from different clinical samples mainly throat, ear, eye, sputum, CSF. The 10 isolates then subjected to further molecular detection method using *P6* as a genetic marker for confirmed isolation of *H. influenzae* by PCR as shown in figure 1 and the results revealed that only 6 isolates out of 10 were positive as shown in table 3.

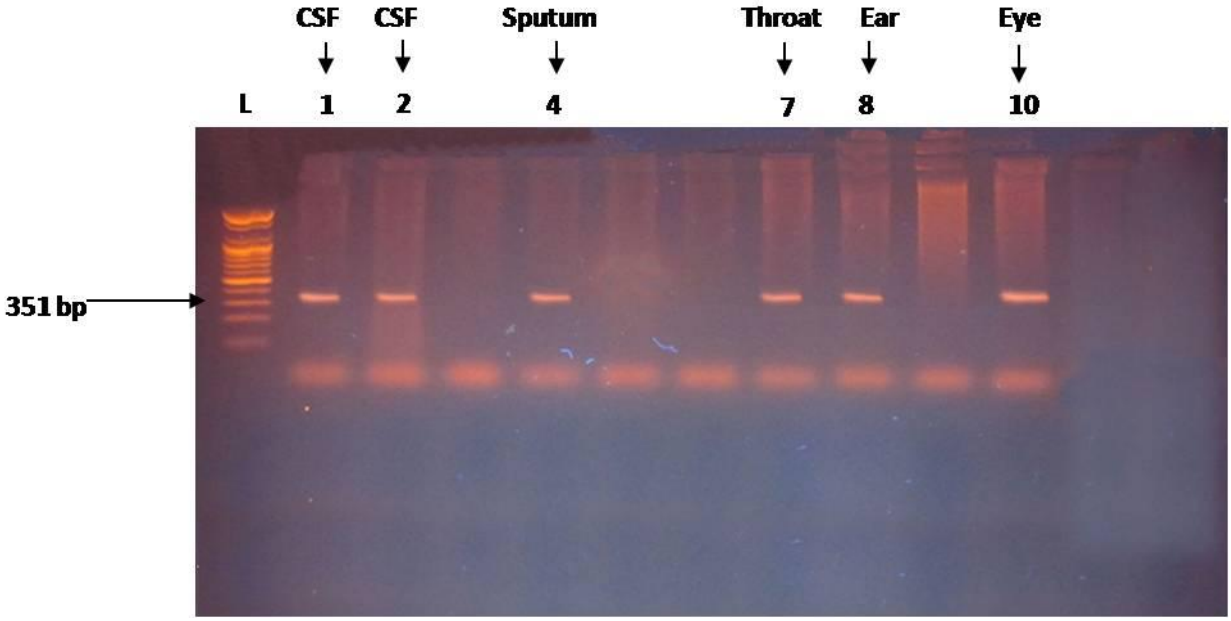

Fig. 1. Gel electrophoresis of PCR product of *P6* gene. Lane of isolates numbered (1,2,4,7,8,10) were positive, where as isolates (3,5,6,9) were negative.

Table 3. Number of isolated *H. influenzae.*

Source of isolates	No. of samples	No. of *H. influenzae* by standard bacteriological method	Using X+V to detect *H. influenzae*	Using PCR by P6 gene
Throat	45	6 (13.3%)	2 (33.3%)	1 (50%)
Ear	50	8 (16%)	1 (12.5%)	1 (100%)
Eye	45	5 (11.1%)	2 (40%)	1 (50%)
Sputum	40	4 (10%)	2 (50%)	1 (50%)
CSF	40	6 (15%)	3 (50%)	2 (66.6%)
Total	220	29 (13.2%)	10 (34.5%)	6 (60%)

All the 6 confirmed isolates of *H. influenzae* underwent testing to separate and differentiate into capsulated (type-able) and non capsulated (non-type-able) depending on the presence or absence of capsule by using specific primers to detect capsule locus of *H.influenzae* namely bexA and bexB. The results revealed that 2 isolates out of 6 (33.3%) were type-able (i.e.,

capsulated) while 4 isolates out of 6 (66.6%) were non-type-able. The results also showed that these 2 capsulated isolates of *H. influenzae* were from CSF of patient with meningitis as shown in figure 2 and 3, respectively.

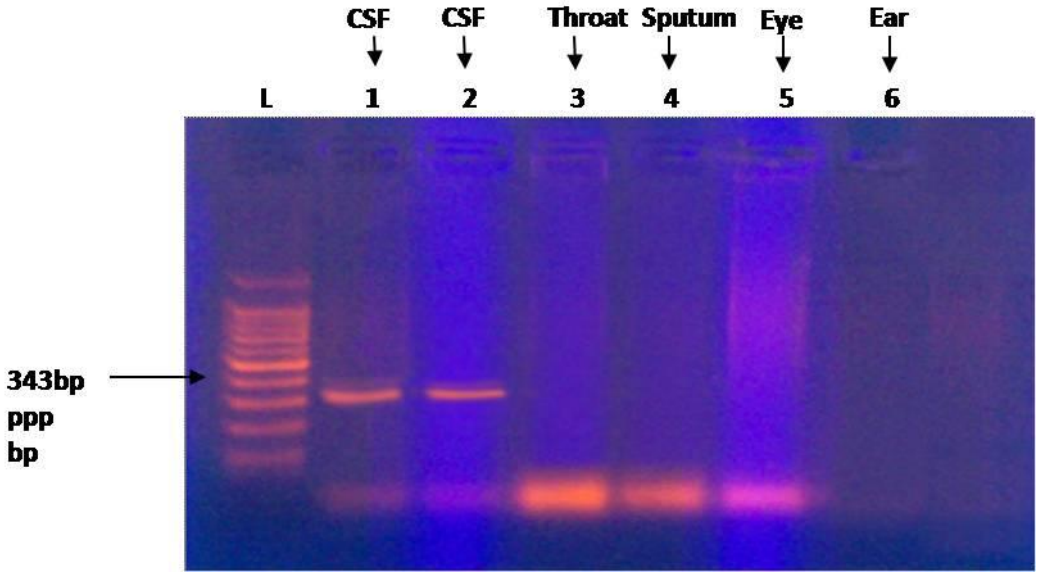

Fig. 2. Gel electrophoresis of PCR product of BexA gene. Lane of isolates numbered (1,2) were positive, where as isolates (3,4,5,6) were negative.

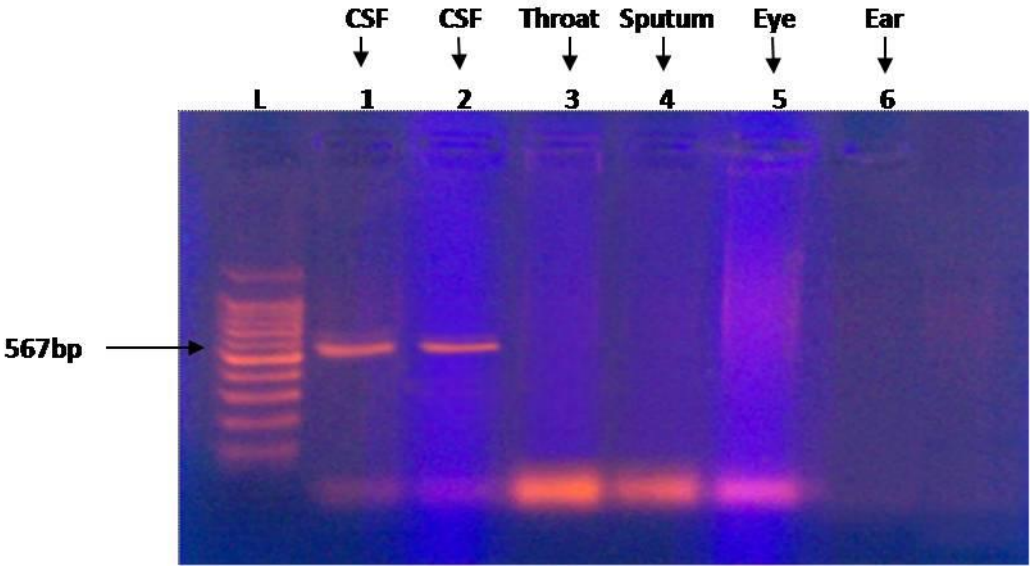

Fig. 3. Gel electrophoresis of PCR product of Bex B gene. Lane of isolates numbered (1,2) were positive, where as isolates (3,4,5,6) were negative.

The remaining 4 isolates 4-6 (66.6 %) give all (100%) positive result for OMP P2 which can be used as a genetic marker for detection of non-type-able one as in figure 4. From the two capsulated one (50%) was of type b H. influenzae by using Hib and bex primers as in figures 5 and 6, respectively.

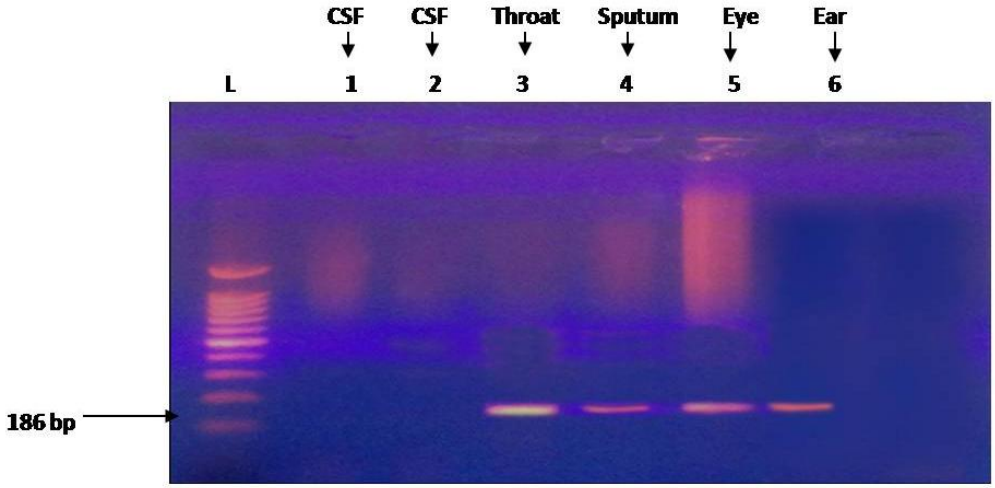

Fig. 4. Gel electrophoresis of PCR product of *P2* gene. Lane of isolates numbered (3,4,5,6) were positive, where as isolates (1,2) were negative.

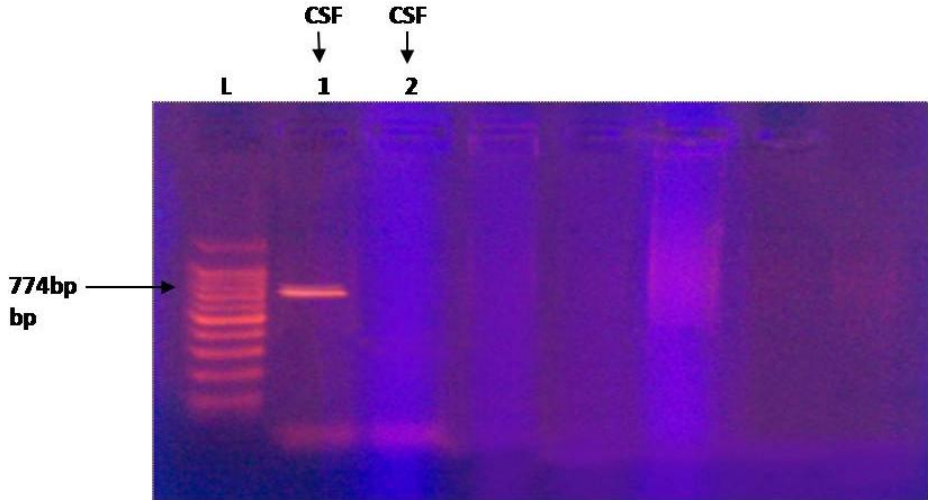

Fig. 5. Gel electrophoresis of PCR product of hib amplicon gene. Lane of isolate numbered (1) was

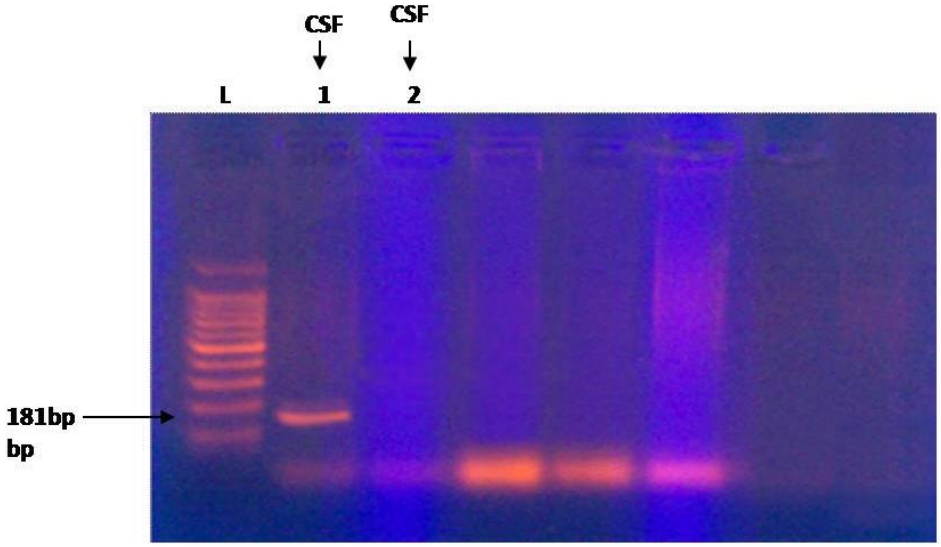

Fig. 6. Gel electrophoresis of PCR product of bex gene. Lane of isolate numbered (1) was positive

Discussion

The results indicate that isolation rate differs according to site of isolation and method applied and this can also be attributed to different virulence factors expressed by *H. influenzae* in different sites of human body as the natural host for it and can be correlated also with the severity and invasiveness of the disease. The results of this study agreed with the results obtained in another study [14] where they detected *H. influenzae* by cultural method and by using X, V discs and specific culture media at a rate of about (18.05%). Also another study found that the isolation of *H. influenzae* is *increased* by using selective media and X, V factors discs and isolated it in a rate of (27.16%) [15]. The same result was described by Mojgani [16] where he isolated *H. influenzae* from CSF of patient with meningitis, eye, mucous from patient with conjunctivitis and nasopharyngeal and ear swabs from patient with otitis media and throat infection, they isolated it at about (31.4%), based on their morphology and growth requirement for X and V factors.

In contrast to the results of this study, other study found 80 isolates of *H. influenzae* on the basis of their growth requirement and serotype distribution [17]. Generally, different isolation rate could also explained by different factors like age, season, the size of the facility, antibiotic treatment, morbidity from acute URIs, the sampling technique ,and the methods that give more accurate sensitivity and specificity. While molecular detection method confirmed the isolation of *H. influenzae* and focused on role of P6 outer membrane protein OMP *P6* gene in this molecular detection method and it detect about 6 isolates (60%) of *H. influenzae* by PCR. Although, *H. influenzae* have many OMPs like P5, P2, but unlik p2 protein, p6 protein show a very high homology (97%) in the amino acid analyses of type b and non-type-able *H. influenzae* strains, which shows that this protein is stable and conserved [3].

Many studies concentrated and focused on this genetic primer and found that *H. influenzae* detection could be achieved with varying degree of success with primers specific for rRNA-encoding genes [18], yet, the rRNA sequences of *H. influenzae* and *H. parainflluenzae* show approximately 95% homology. So, these genes are not ideal targets for the unequivocal identification of *H. influenzae* [19]. Previous studies had shown that NTHi OMP P6 had 100% homology among human respiratory isolates from adults and children [20]. Regarding the determination of capsular type ability by molecular methods detection, the result revealed that 2 isolates out of 6 (33.3%) were type-able (i.e., capsulated) while 4 isolates out of 6 (66.6%) were non-type-able.

The results also showed that these 2 capsulated isolates of *H. influenzae* were from CSF of patient with meningitis and this is accepted regarding the presence of capsule that make it resistant to macrophage, complement and human defense and provides the ability to be invasive and so cause severe disease and even make it resistant to many antibiotics. The highly conserved *bexA* and *bexB* genes, which are required by type-able strain for the transport of capsule components across the outer membrane, were assayed by PCR following the protocol of Davis [21].The advantage of this method over tranditional slides agglutination techniques using type-specific antisera or methods detecting *bexA* alone is that *bexB* PCR will detect rare strains that are *bexA* negative but *bexB* positive, which render them phenotypically non-type-able but genetically far closer to type-able strains [22].

Another studies used PCR reaction to detect *bexA* gene and found that its absence or presence determined whether` an isolate was encapsulated or non capsulated, their study revealed a rate of NTHi to be about 93.5% while type-able one was (6.45%) distributed into different serotypes from a-f [23]. So our results differed from this study and this may be attributed to dependence of their study on *bexA* alone and also their isolates were mostly nasopharyngeal isolates. Many studies found that differentiating type-able from non-type-able *H. influenzae* strains can be challenging

where type-specific serum agglutination had classically been used to confirm the presence and specifity of *H. influenzae* capsule. However, a strain may fail to react with typing sera and thus be classified as non-type-able for several reasons [24].

First: inaccuracy in performing and interpreting slide agglutination tests had been well documented.

Second: strain with one copy of the *cap* region in which *bexA* is partially deleted is referred to as capsule deficient variants because they contain a majority of the *cap* locus.

Third: a previously serotypeable strain could have a deletion of the entire capsule locus, as apparently occurred with strain Rd (non- type-able variant of type d strain).

Fourth and finally: a strain may lack the entire *cap* locus as consequences of long-past evolutionary events, i.e., may be a true NTHi strain [25]. Davis [21] found that *bexB* which is located adjacent to *bexA* in region I of the capsule locus and encodes another protein important in capsule exportation, is a more reliable marker of the capsule locus because it can be detected in *H. influenzae* strains that posses a single *cap* locus and a *bexA* mutation in that locus. Another study done by Mojgani et al [16] isolated *H. influenzae* at a rate different from the rate of this study where NTHi isolation rate was (38.6%). While type-able one was (61%) by using *bexA* primer and found that most of NTHi isolates were from nasopharyngeal secretion while type-able *H. influenzae* were mostly from CSF. The results of this study are in contrast with the results of other studies where they identified and isolated NTHi at a rate of about (33%) while type-able one was (66.6%) but most of their isolates were from invasive *H. influenzae* disease *(26)*. Many studies proposed that the ancestor of *H. influenzae* was encapsulated and the non-type-able clones arose by convergent evolutionary loss of the ability to synthesize or extracellularly express a polysaccharide capsule.

However, the wide heterogenecity of non-type-able strains, the more clonal features of type-able strains and the evidence that most type b specific genetic regions are flanked by repeat sequences and thus may represent acquisition of foreign genetic elements, make it more likely that an un encapsulated ancestral *H. influenzae* strains acquired these elements and became more virulent [27].

While the detection of non-type-able H. *influenzae* at 1st by absence of capsule gene and by P2 gene that is conserved for them. In this study it was used to differentiate between both type-able and NTHi and since it expresses on NTHi so it is possible to confirm that the remaining 4 isolates that was non-capsulated by using Bex primers (A, B) were non-type-able because some isolates that have mutation or deletion or single copy of *cap locus* sometimes fail to express capsule and can be regarding as non- capsulated, but these isolates are not true NTHi.

The emerging role of invasive disease because NTHi is intriguing because this organism had traditionally been considered relatively non-invasive bacteria predominantly associated with community-acquired pneumonia, chronic obstructive pulmonary disease exacerbations and otitis media [28]. Regarding the Detection of type b *H. influenzae*, the study revealed that two isolates were of capsulated (type-able) *H.influenzae and* further primers were used to detect the serotypes of these two isolates and specifically type b (Hib) since these two isolates were from CSF of patient with meningitis and so type b could be the causative agent among these isolates. However, the results showed that only one isolate was type b (Hib) while the other one was capsulated non type b *H. influenzae*.

In this study two primers were used, one called bex to detect Hib from CSF of patient with meningitis specifically and to exclude *N. meningitis* and *S. pneumoniae* while the other one called Hib which confirmed the results and both gave the same results. Center for Disease Control and Prevention [29] revealed that infection with Hib can cause meningitis in 50% of cases of adults and children and this results are similar to the results of this study and they also

revealed to be more prevalent in developing countries. Other serotypes could also be isolated from invasive infection where other studies isolated type a (48%), f (14%) d (5%), c (2%) [26]. Furthermore, the association of non-b-capsular serotypes with invasive disease could be facilitated by the acquisition of virulence factors common to Hib such as capsule gene duplication and an IS1016-bex A deletion in the capsule gene cluster which may serve to stabilize capsule production [25]. In addition, infrequent recombination, event could happen between naturally transformable Hib and other serotype that may enhance the fitness and virulence of these serotypes [30].

We can conclude from the current work that using of specific genetic marker namely P6 primer is important in molecular detection of both types of *H. influenzae* that isolated from different sites. Detection of capsule is valuable in differentiation of type-able from non-type-able one. Non-type-able *H. influenzae* (NTHi) is also an important cause of invasive and severe disease like upper and lower respiratory tract disease and eye infection. Using serotype specific gene is necessary among patient with meningitis since not only type b (Hib) can cause meningitis where other capsulated non b *H. influenzae* could also implicate.

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Correspondence to Dr. Bara H. Hadi
E-mail: myanBara@gmail.com
Mobile: + 964 7803414404
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Provocative Test's *Versus* Electrophysiological Studies as a Measure of Severity Grades of Carpal Tunnel Syndrome

Zaki N. Hasan[1] *FICMS*, **Safaa H. Ali**[2] *PhD*

[1]Dept. of Medicine, Al-Kindy College of Medicine, Baghdad University, [2]Dept. of Physiology, College of Medicine, Al-Mustansiryah University, Baghdad, Iraq.

Abstract

Background Carpal tunnel syndrome (CTS) is the most common nerve entrapment, electrodiagnostic studies are a valid and reliable means of confirming the diagnosis.

Objectives The study aims to find a correlation between the presence of Tinel's sign and Phalen's maneuver and the degree of severity of the CTS and to compare it with severity of nerve conduction study of median nerve.

Methods The study involves 133 patients (102 females and 31 males) with CTS, all were examined for Phalen's maneuver and Tinel's sign and median and ulnar nerves electro physiological study in Al-Yarmouk Teaching Hospital and the Neurosciences Hospital in Baghdad between January 2010 and January 2011. Their ages ranged between (19-87) years. The patients were grouped into mild, moderate and severe CTS according to modified Padua scale of CTS severity. Statistical correlation was done using one way Anova test.

Results Positive Tinel's sign was seen in 25% and positive Phalen's maneuver in 28%, coexistent Tinel's sign and Phalen's maneuver positive at the same time were seen in 47%. Total Tinel's sign was72% and total patients who had positive Phalen's sign was 75%. Mild, moderate and severe CTS were seen in 38%, 41% and 21% out of the total number of the studied patients.

Conclusion The study didn't find association between severity grading and provocative test, added to negative provocative tests in high percentage of patients. These results mandate the use of electrophysiological examination for the diagnosis of carpal tunnel syndrome and assessment of severity.

Keywords Carpal tunnel syndrome, Tinel's sign, Phalen's maneuver

Introduction

Carpal tunnel syndrome (CTS) is the commonest entrapment neuropathy which is characterized by a combination of clinical symptoms and signs arisen from compression of the median nerve at the wrist [(1)]. It is characterized by tingling, numbness and pain in the first three fingers and half the ring finger of the hand, it is commonly radiating to the forearm [(1,2)]. Diagnosis of CTS is based on clinical symptoms, physical signs, and nerve conduction abnormalities [(3)].

Diagnosis based only on symptoms or signs are less reliable because other common disorders such as tendonitis and cervical radiculopathy may cause similar symptoms and signs. Thus, electrophysiological testing is often employed to confirm the clinical diagnosis.

Electrophysiological findings, includes abnormal sensory conduction over the tested segments

and prolonged terminal sensory and motor latencies. With more severe CTS cases, electrodiagnostic study usually shows some secondary axonal loss reflected in reduced amplitude and area of the compound muscle action potential (CMAP) in response to the stimulation at any point along the nerve [3].

Phalen's maneuver and Tinel's sign are the most useful clinical signs for diagnosis of CTS. Tinel's sign elicited by tapping over the median nerve at wrist leading to tingling sensation in the distribution of the median nerve over the hand.

Phalen's maneuver was done by holding the wrist passively flexed for 30 seconds to 2 minutes, it was considered positive when leads to tingling sensation in the distribution of the median nerve over the hand [4].

The accuracy of the diagnosis of CTS is important because the diagnosis often leads to surgical release of the carpal ligament in patients whose symptoms are refractory to non-operative therapy. If the symptoms are not due to CTS, then the patient is unlikely to benefit from surgery [2].

We aim to find a correlation between the degree of severity of the CTS by nerve conduction study of median nerve and the presence of Tinel's sign and Phalen's maneuver and eventually if we can assess severity only by assessment of provocative tests.

Methods

A cross-sectional study enrolled 133 patients (102 females and 31 males) referred to Al-Yarmouk Teaching Hospital and the Neurosciences Hospital between Jan 2010 and Jan 2011 with hand complaints compatible with CTS and approved by electrophysiology as a CTS. Their ages ranged between (19-87) years. Seventy-five of the patients had left sided complaints and 58 had right sided complaints, we studied only the affected side. The patient verbal consent to be involved in the study was taken. Owing to the study is clinical one; it doesn't need an ethical approval.

The criterion for inclusion were clinically and electrophysiological proven CTS patients. The criteria for exclusion were clinical or electrophysiological evidence of generalized peripheral neuropathy, evidences of cervical radiculopathies and any diseases leading to peripheral polyneuropathies such as diabetes mellitus, renal disease and rheumatologic diseases.

Clinical assessment was first done for each patient with special emphasis on Tinel's sign; which considered positive when taping over the median nerve at the wrist leads to tingling sensation in the distribution of the median nerve over the hand. Phalen's maneuver was done by holding the wrist passively flexed for 1 minute, it was considered positive when leads to tingling sensation in the distribution of the median nerve over the hand.

Immediately thereafter, an electrophysiological study was done to prove the diagnosis of CTS. Nerve conduction studies were performed using standard techniques of supramaximal percutaneous stimulation with a constant current stimulator and surface electrode recording, maintaining skin temperature 32°C.

Sensory responses were obtained anti-dromically, stimulating at the wrist and recording from the index finger (median nerve) and little finger (ulnar nerve), with ring electrodes at a distance of 14 cm. Motor responses were obtained with stimulation at the wrist using belly-tendon recordings from the thenar muscles (median nerve) and hypothenar muscles (ulnar nerve) at a distance of 7 cm.

Sensory conduction velocity was the distal conduction velocity, determined by dividing the wrist-to-electrode distance (14 cm) by the distal onset latency of the sensory nerve action potential. For this study, the following median nerve measures were used:

(1) baseline-to peak amplitude of the sensory nerve action potential (Amp-S);

(2) distal onset latency of the sensory nerve action potential (DL-S);

(3) conduction velocity of the sensory nerve fibers (CV-S);

(4) baseline-to-peak amplitude of the compound muscle action potential (Amp-M); and

(5) distal onset latency of the compound muscle action potential (DL-M).

Carpal tunnel syndrome was defined as being present when ulnar nerve studies were normal and median nerve studies met one of the following criteria for abnormality based on normal values obtained and used in our laboratory: (1) DL-S > 3.7 ms; (2) DL-M > 4.4 ms; and (3) CV-S < 49 m/s.

All subjects were investigated for sensory nerve conduction velocity (SNCV) of affected and unaffected side median nerves and the same side ulnar nerve. Amplitude, SNCV and distal sensory latency were assessed antidromically using a pair of ring surface electrodes on the index finger and little finger.

Motor nerve conduction of affected and unaffected side median nerves and the same side ulnar nerve using surface electrode were also assessed. Also needle EMG study of affected side abductor polices and abductor digit minimi muscles were done.

Examination was done with EMG/NCS apparatus Micro Med with setting for sensory studies were: Frequency: 100 Hz-10KHz. Sweep speed: 2 ms/Division. Sensitivity: 10 µV/Division, and for motor studies was: Frequency: 100-500 Hz. Sweep speed: 5 ms/Division. Sensitivity: 200 µV/Division. Diagnosis of CTS was based on the criteria of the American Association of Electrodiagnostic Medicine (AAEM) on getting 2 out of 3 following criteria [5].

1) Antidromic sensory conduction velocity for index digit segment less than 48.2 m/sec.

2) The difference between median and ulnar sensory nerve distal latencies with recording from the fourth digit (recording-stimulation distance was kept 14 cm) exceeding 0.5 ms.

3) Distal motor latency to abductor pollicis brevis muscle greater than 4.2 ms.

CTS severity was classified into mild, moderate and severe CTS according to the modified Padua Criteria [6]: Mild CTS: Prolongation of median distal sensory latency > 3.5 ms or relative prolongation of median compared to ulnar distal sensory latencies over identical distances. Moderate CTS: Reduced median SNAP amplitude (< 50% compared to unaffected side or < 10 µV are considered abnormal) or prolonged median motor distal > 4.5 ms.

Severe CTS: Reduced median CMAP amplitude (< 50% compared to unaffected side or < 4 mV), denervation of median innervated muscles on needle exam. After that the results of the presence of the Tinel's and Phalen's sign were correlated with each step of severity of the CTS using one way Anova test. Then each step of severity was correlated with presence of the provocative tests using one way Anova test.

Statistical analysis was done using graph pad software (Quick calc online calculator for Scientist) with P value less than 0.05 was the cutoff point of significant differences.

Results

Isolated Positive Tinel's sign only was seen in 33 out of 133 (25%) and Positive Phalen's maneuver only in 37 out of 133 (28%). Coexistent Tinel's sign and Phalen's maneuver positive at the same time were seen in 63 out of 133 (47%), (See Table 1). So the total patients who had Tinel's sign was 96 (33+63) out of 133 hands (72%) and total patients who had positive Phalen's sign was 100 (37+63) out of 133 (75%). Mild, moderate and severe CTS was seen in 51/133 (38%), 54/133 (41%) and 28/133 (21%) out of the total number of the studied patients. Both signs positive was seen in 25/63 (40%), 26/63 (41%), and 12/63 (19%), in mild, moderate and severe CTS respectively.

Table 1. The percentage of Tinel's and Phalen's signs

Provocative test	Total
+ ve Tinel's sign only	33 (25%)
+ ve Phalen's only	37 (28%)
Both + ve at the same time	63 (47%)
Total	133

Phalen's sign only was seen in 12/37 (32%), 15/37 (41%) and 10/37 (27%) in mild, moderate and severe CTS respectively. Tinel's sign only was seen in 14/33 (42.3%), 13/33 (39.4%) and

6/33 (18.2%) in mild, moderate and severe CTS respectively (Table 2 and 3).

Discussion

Carpal tunnel syndrome affects almost 5% of the population and is most common in middle-aged women, in about 70% of the cases; it is bilateral and is prevalent in the dominant hand.

The sensitivities of all the provocative tests are different according to the levels of electrodiagnostic severity [7]. In the present study Positive Tinel's sign only was seen in (25%) and Positive Phalen's maneuver only in (28%). Both Tinel's sign and Phalen's maneuver positive at the same time were seen in (47%). Total Tinel's sign was seen in 72% and total Phalen's maneuver positive was seen in 75%. These results is higher than the results of other studies that showed 62% and 45% of carpal tunnel syndrome had Tinel's sign and positive Phalen's test respectively. Phalen found a positive Tinel's sign in 73% of hands of patients with CTS [8]. Stewart et al. and Gelmers *et al*. studies found Tinel's sign was seen in approximately 45% of their patients [9,10].

Table 2. Correlation of CTS severity with provocative tests of CTS

CTS severity	+ ve Both signs	+ ve Phalen's sign	+ve Tinel's sign
Mild	25/63 (40%)	12/37 (32%)	14/33 (42.3%)
Moderate	26/63 (41%)	15/37 (41%)	13/33 (39.4%)
Severe	12/63 (19%)	10/37 (27%)	6/33 (18.2%)
Total	63/63	37/37	33/33

P = 0.577

Table 3. Correlation of provocative tests with the CTS severity

Provocative Test	Mild	Moderate	Severe	Total
Tinel's Sign	25/51 (49%)	26/54 (48%)	12/28 (43%)	63
Phalen's sign only	12/51 (23.5%)	15/54 (28%)]	10/28 (36%)	37
Both signs positive	14/51 (27.5%)	13/54 (24%)	6/28 (29%)	33
Total	51/133 (38%)	54/133 (41%)	28/133 (21%)	133

P = 0.949

The results of phalen`s sign in the present study was 75%; which is in approximate to the results of numerous studies of Phalen's maneuver in the hands of patients with suspected carpal tunnel syndrome which varied from 10% to 88%, with an average of 62% [11,12]. The present study showed 38% of CTS was mild, 41% moderate and in 21% it was severe. This is different from Yazdchi *et al*. study who`s percentage of moderate and severe severity groups were 53.8% and 13.5% respectively.

The higher rate of severe group and the lower rate of moderate severity group in the present study was related to poor awareness of the disease and late seeking of medical consultation until reaching severe pain in Iraqi patients [13].

The present study results showed no correlation between presence of provocative signs whether phalen`s sign or tinel`s sign with steps of severity according to modified Padua scale of CTS severity; this results was not agreeing the conclusions of Italian CTS study group and Bland study whom demonstrated a good correlation between the clinical and electrophysiological staging of the CTS [14,2].

Also this result is not agreeing Ahn *et al*. study who found that Provocative tests have little merit as diagnostic tools in "severe" and "mild"

cases of CTS but the provocative tests are much more reliable in "moderate" cases [15].

Based on the above results of the present study of no correlation between presence of provocative signs whether phalen`s sign or tinel`s sign with steps of severity of electrophysiological study and presence of positive phalen`s sign and Tinel`s sign in 20 % and 25% of healthy peoples respectively [12,16], hence those electrophysiological studies is mandatory for diagnosis as well as for severity categorization; furthermore many reports suggested that the neurophysiologic finding of carpal tunnel syndrome has superior sensitivity compared to the clinical sign of the disease; so that the provocative tests often are negative in spite of obvious presentation of the syndrome and evident electrophysiological abnormalities of the disease [14].

In conclusion, provocative tests of carpal tunnel syndrome is not elicited in good percentage of patients, no correlation between presence of provocative signs whether phalen`s sign or tinel`s sign with steps of severity of electrophysiological study and there is no correlation between severity of electrophysiological study and presence of provocative signs.

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Correspondence to Dr. Safaa H. Al-Shammary
E-mail: safaalshammary@yahoo.com.
Mobile: + 964 7904884960)
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Expression of P53 Protein in Neoplastic and Non Neoplastic Ovarian Lesions

Fahem M. Mahmood[1] *BSc*, **Haider S. Kadhim**[2] *PhD*, **Liqaa R. Al Khuzaee**[3] *FIBOG*

[1]Dept. of Pathology, Dept. of [2]Microbiology, [3]Dept. of Obstetrics & Gynecology, College of Medicine, Al-Nahrain University

Abstract

Background Ovarian cancer is one of the most common causes of gynecologic neoplasm all over the world.

Objective The objective is to shed light on the role of p53 protein and patient`s age in the pathogenesis of ovarian lesions.

Methods Paraffin embedded blocks of 62 patients with ovarian lesions were studied. Thirty-five cases of surface epithelial ovarian tumors, (31 cases of invasive surface epithelial ovarian tumors, and 4 cases of borderline intermediate malignancy cases of neoplastic ovarian cystic lesions). In addition, eighteen cases of benign neoplastic ovarian cystic lesions and 9 cases of non- neoplastic functional one were enrolled in this study. All of cases included, were stained with p53 by immunohistochemistry.

Results Immunohistochemistry for p53 showed that malignant cases were positive for p53 while all benign cases were negative for p53 and the borderline cases were also negative for p53. The non-neoplastic cases were negative for p53. There is a significant statistical difference in P53 expression in malignant cases compared to other groups ($P < 0.001$). A significant difference in mean age of malignant and border line cases in comparison with benign and non-neoplastic cases; ($P < 0.001$).

Conclusion Protein p53 may play a role in the pathogenesis of malignant ovarian cancer but not in benign lesions. The age of the patient has a role as a risk factor in ovarian lesions.

Keywords Ovarian lesion, ovarian cancer, p53, immunohistochemistry.

Introduction

Ovarian cancer is one of the most common causes of gynecologic neoplasm and is the fifth cause of cancer mortality in women. The high mortality rate in women with ovarian cancer is due to its detection at advanced stages.

Even though there have been improvements in surgical techniques and treatment options, five-year survival for ovarian cancer still remain at approximately 45% [(1)].

Ovarian tumors are heterogenous. Insight into their pathogenesis requires understanding of the mutations involved, overexpression of oncogenes and role of cell cycle regulators. There have been persistent efforts in the investigation of molecular markers in epithelial ovarian tumors, but the results are controversial [(2)].

Among the most common genetic alterations in human ovarian cancer are p*53* mutations. Defects in this tumor suppressor pathway are

present in over eighty percent of human cancers [3,4] and have been associated with poor prognosis in ovarian carcinomas [5,6].

Mutation in the p53 gene is the most common single genetic alteration in human ovarian cancer. Either loss of wild type p53 protein function, gain of oncogenic function or the ability to activate p53 protein inappropriately severely compromises the capacity for controlled cellular proliferation and cellular growth [7].

A number of studies that have paid particular attention to histological criteria of malignancy of serous tumors have found that p53 mutations are strongly associated with high-grade serous carcinomas, but are rare in low grade or borderline serous carcinomas [8-11]. The p53 protein plays a key role in cell cycle regulation and suppression of tumor development. DNA damage results in increased levels of p53, which lead to cell cycle arrest in G1 phase, followed by DNA repair or apoptosis. Mutations of the p53 gene as determined by mutation analysis and/or positive immunohistochemical (IHC) staining for p53 are common in ovarian cancer and have been associated with poor clinical outcome [12].

The aim of the current study is to evaluate the expression of p53 by immunohistochemistry (IHC) and to compare it with clinicopathologic prognostic factors of ovarian tumors namely age and malignancy.

Methods

In this study, 62 ovarian cystic lesions were involved. Specimens belong to the period from June 2011 to March 2012 were collected from private laboratory in Baghdad. According to the hematoxylin and eosin staining, the patients were grouped into:

- Thirty five cases of surface epithelial ovarian tumors, (31 cases of invasive surface epithelial ovarian tumors, and 4 cases of borderline intermediate malignancy cases of neoplastic ovarian cystic lesions).
- Eighteen cases of benign neoplastic ovarian cystic lesions .
- Nine cases of non- neoplastic functional one.

The diagnosis of these tissue blocks was based on the obtained pathological records of these cases from laboratory records. Following processing of these tissue blocks, a confirmatory histopathological re-examination of the slides was done by consultant histopathologist in Department of Pathology, College of Medicine, Al-Nahrain University.

Sections were made from each of the paraffin embedded blocks as follows: one section 4 µm thick sections were made on ordinary slides to be subjected to haematoxylin and eosin stain. This was conducted to confirm the diagnosis and tumor grade. Another section, 4 µm thick sections was made on positively charged slide for detection of p53 by immunohistochemistry using monoclonal mouse Anti human p53 protein. This technique is done the Department of Pathology and Department of Microbiology College of Medicine Al-Nahrain University. It is based on the detection of the product of gene expression (protein) in malignant and normal cells using specific monoclonal antibodies, i.e., primary antibody for specific epitope, which binds to nuclear targeted protein. The bound primary antibody is then detected by secondary antibody (usually rabbit or goat anti-mouse), which contains specific label (in this context we used peroxidase labeled polymer conjugated to goat-anti mouse immunoglobulin). The substrate is peroxidase (H_2O_2) in diaminobenzedine (DAB) of chromogen solution then stained with hematoxlyin as a counter stain. Positive reaction will result in a brown colored precipitate at the antigen site in tested tissue [13] shown in figure 1.

Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007. Nominal data were expressed as frequency and percentage. Numeric data were expressed as mean $\pm$SEM (Standard error of mean). Chi-square test was used to assess relation between nominal data, while ANOVA test and student t-test were used to analyze difference among the mean of numeric data. *P*-value (< 0.05) was considered significant.

Results

The current study included four major categories: malignant, borderline, benign and non-neoplastic cases. The non-neoplastic cases include 4 (50%) corpus luteum cysts and 4 (50%) follicular cysts.

The benign cases include: 9 (50%) serous adenoma, 9 (50%) mucinous adenoma. The borderline cases include: 2 (50%) serous tumors, 1 (25%) mucinous tumors and 1 (25%) endometroid tumors. The malignant cases include: 26 (83.87%) serous tumors, 2 (6.45%) mucinous tumors and 3 (9.68%) endometroid tumors as seen in table 1.

Table 1. Neoplastic cases

Type	Benign		Borderline		Malignant		Total	
	No.	%	No.	%	No.	%	No.	%
Serous	9	50	2	50	26	83.87	37	69.81
Mucinous	9	50	1	25	2	6.45	12	22.64
Endometroid	0	0	1	25	3	9.68	4	7.54
Total	18	100	4	100	31	100	53	100

The mean age of non-neoplastic cases is (34.0±4.46) years. While, the mean age of benign cases is (31.44±1.96) years. In addition, the mean age of borderline cases is (51.75±4.3) years and the mean age of malignant cases is (50.06±1.86). There is a significant difference in mean age of malignant and borderline cases in comparison with benign and non-neoplastic cases; ($P < 0.001$) as shown in table 2.

Table 2. Comparison of mean age among the neoplastic and non-neoplastic cases

Group	No.	Mean Age	SEM
Non neoplastic	9	34.00	4.46
Benign	18	31.44	1.96
Borderline	4	51.75	4.30
Malignant	31	50.06	1.86

$P = < 0.001$

Malignant cases showed positive result in 30 cases out of 31 for P53. While the benign cases (18 cases) were negative for P53. Moreover, the borderline cases (4 cases) were negative for p53 and the non-neoplastic cases (9 cases) were also negative for p53, (Table 3).

Table 3. Immunohistochemical expression of P53

P53 Results	Non neoplastic		Benign		Borderline		Malignant	
	No.	%	No.	%	No.	%	No.	%
Positive p53	0	0	0	0	0	0	30	96.8
Negative p53	9	100	18	100	4	100	1	3.2
Total	9	100	18	100	4	100	31	100

$P = < 0.001$

There is a significant statistical difference in p53 expression in malignant cases compared to other groups ($P < 0.001$).

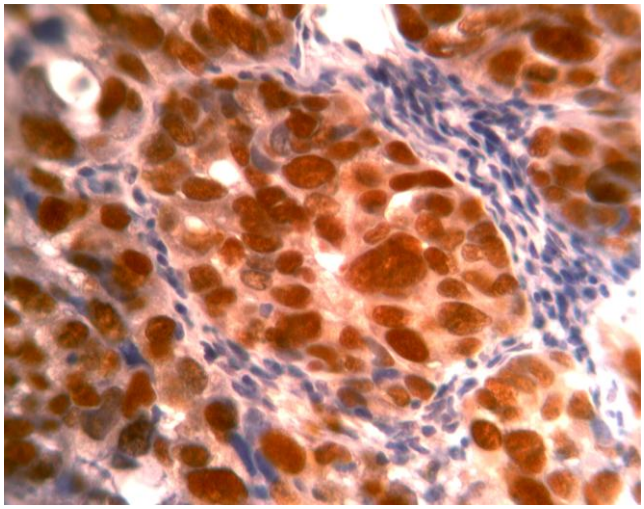

Figure 1: Moderately differentiated ovarian serous cystadenocarcinoma showing intense nuclear P53 staining reaction DAB staining (200 X)

Discussion

Ovarian cancer is the seventh most common cancer in women worldwide, with nearly a quarter of a million women diagnosed every year. 5-year survival is just 30%, a figure that has not changed for the past 30 years [14]. The current study showed that p53 was negative in all benign tumors but positive in 96.8% of malignant cases. Previous studies showed mutation in or inactivation of p53 in 57% [2], 46% [15] of invasive ovarian tumors, but in only 8% of borderline tumors and nonexistent in benign tumors [16]. This difference with the results of the current study may be due to genetic background differences, samples size or methodology variations.

Alterations of p*53* occur via a variety of mechanisms, such as mutations and deletions, or protein stabilization without any obvious genetic changes. Point mutations often result in a dominant-negative inhibition of the function of the wild type allele and/or gain of novel functions. Most of these mutant p53 proteins have a prolonged half-life, accumulate in the nucleus and can be detected by immunohistochemistry [17] whilst 26% to 81% of ovarian cancers have been reported to have mutations or overexpression of p*53* [18].

In ovarian cancer, the age of the patient considered an important risk factor. Patients older than 69 years of age exhibited significantly poorer survival than those younger [18]. In the current study, there was a significant difference in mean age of malignant and borderline cases in comparison with benign and non-neoplastic cases.

Different studies [12,19-21] showed over-expression of p53 detected by immunohistochemistry, as Kerbel *et al* [20] showed that p53 overexpression was detected in 43.3% of serous ovarian cancer while none of the normal ovarian tissues revealed immunohistochemical expression for p53, with significant level of expression between malignant and benign tissues ($p< 0.001$). p53 overexpression was reported more frequently in higher grades of differentiation with significant level of expression ($P < 0.05$) this indicates that serous ovarian tumors with positive p53 expression are biologically bearing more aggressive behavior and patient`s age both can be used as a prognostic markers in patient with ovarian cancer [20].

Another study done in Mosul/IRAQ by Hamdi and Saleem [21] in 2012, showed that, p53 expression was not significantly related to the age of the patients, grade, or to the histological type of the tumors. It was mainly found in malignant serous tumors (50%), in the poorly differentiated tumors (47.6%), and in the 6th decade of age (30.8%) [21].

In conclusion, p53 may play a role in the pathogenesis of ovarian cancer, in addition to patient`s age.

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Correspondence to Dr Haider S. Kadhim
Email: haider_kadhim@yahoo.com
P. O. Box 70056, Al-Kadhimiya, Baghdad, Iraq.
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Effect of Breastfeeding during Pregnancy on the Occurrence of Miscarriage and Preterm Labour

Maysara M. Albadran *CABOG, FICMS, MRCOG*

Dept. of Obstetrics & Gynaecology, College of Medicine, Basrah University, Iraq

Abstract

Background Many mothers breastfeed their babies while they are pregnant.

Objective To explore whether breastfeeding during pregnancy increases the risk of miscarriage and preterm births.

Methods A case-control study conducted in Al-Mawany General Hospital through a period extended from first of September 2011 till the first of September 2012. Two hundred fifteen pregnant women who breastfed during pregnancy and two hundred eighty pregnant who weren't breastfeeding during pregnancy were studied. Demographic data, frequency of miscarriage and preterm deliveries and neonatal birth weight were compared between the two groups. Chi-Square and student *t*-test were used to compare the result. Significant difference was considered when P value < 0.05.

Result The frequency of miscarriage among those who breastfed their babies during pregnancy were significantly lower than among those who didn't breastfed during pregnancy, this was unaffected by exclusiveness of breastfeeding, however, there was statistically insignificant difference in the frequency of preterm deliveries and in the birth weight between the two groups.

Conclusions Breastfeeding doesn't increase the risk of miscarriage or preterm births neither does it affect neonatal birth weight.

Keywords Miscarriage, breastfeeding, preterm labour, exclusive breastfeeding, non exclusive breastfeeding

Introduction

Breastfeeding is the direct feeding of an infant or young child from female breasts rather than from a bottles [(1)] has a lot of benefits for the infant with regard to general health, growth and development. It decreases lower respiratory infections, ear infection and necrotizing enterocolitis, the incidence of sudden infant death syndrome, type I and type II diabetes mellitus, allergic disease (atopy); and possibly enhance cognitive development.

Breast milk contains secretory IgA antibodies, which decrease the incidence of gastroenteritis. Some studies suggest that breastfeeding may decrease the risk of cardiovascular disease in later life, as indicated by lower serum cholesterol in adult women who had been breastfed as infants [(2-5)].

Breastfeeding has a lot of benefits for the mother including:

- Decreases the risk of breast cancer, ovarian cancer, and endometrial cancer [(6-9)].
- Lactation for at least 2 years reduces risk of coronary heart disease by 23% [(10)].
- Decrease insulin requirement in diabetic mothers [(11)].
- Lower risk of metabolic syndrome [(12)].

- Lower risk of post-partum bleeding [13].
- Long duration of breast feeding decrease risk of rheumatoid arthritis [14].

The World Health Organization (WHO) recommends breastfeeding exclusively in the first six months and with complementary foods while breastfeeding continues for up to two years of age or beyond" [15]. Breastfeeding a child during pregnancy consider a type of tandem feeding for the nursing mother as, she provides nutrition for two [16].

The question that frequently asked by the mothers: can I continue breastfeeding while I am pregnant? Does it hurt my pregnancy? Since suckling during breastfeeding induce release of oxytocin from posterior pituitary gland. Oxytocin causes contraction of myoepithelial cells around the mammary gland which enhance milk expulsion [17]. Hence; released oxytocin, theoretically, may cause uterine simulation during pregnancy increasing the risk of miscarriage and preterm labour.

Miscarriage: is the spontaneous end of a pregnancy prior to viability. By 6 weeks gestation, the rate of miscarriage is one in five pregnancy and by the second trimester the rate fallen to 1 in 40. Chromosomal abnormalities present in 50-70% of first trimester miscarriage, other causes include uterine abnormalities, genital tract infection, maternal diseases as thyroid diseases and diabetes mellitus and certain drugs [18].

Preterm birth: is the delivery of a baby of less than 37 completed weeks gestational age [1]. The neonatal mortality or survival with handicap becomes significant in very preterm infants, those born between 28 and 32 weeks, and is most significant in extremely preterm infants, those born before 28 weeks. The incidence of preterm birth in developed world is 7-12%.

The intention of the study is to determine whether there is association between breastfeeding during pregnancy and increase in the risk of miscarriage and preterm birth.

Methods

A case-control study was conducted in Al-Mawany Hospital extended through a period of one year from first of September 2011 till the first of September 2012.

A total of 215 pregnant women with history of breastfeeding during the current pregnancy were studied and compared with a control group of 280 non breastfeeding pregnant. The studied women were either in labour and were collected from labour ward or were with inevitable miscarriage and were collected from the emergency unit.

Full history was taken from each of the studied women including: age, gravidity, previous history of miscarriage or preterm delivery, their gestational age (determined by the date of last menstrual period and early ultrasound record), their past medical history.

Women at extremes of age (less than 18 and more than 35 years old); women with medical diseases as diabetes mellitus, thyroid diseases and sickle cell anaemia, those with history of recurrent miscarriage and preterm deliveries and those with multiple pregnancies were excluded from the study.

Pregnant who breastfed during pregnancy were divided according to the duration of breastfeeding into two groups: those who breastfed for the first 24 weeks of gestation and those who breastfed more than 24 weeks of gestation. The first group were further subdivided according to the type of breastfeeding: exclusive and non exclusive and the occurrence of miscarriage were recorded. Among the second group the duration of pregnancy (term or preterm) and birth weight were measured.

Discrete variables were expressed as numbers and percentages, continuous variables expressed as mean ± standard deviation. Chi-Square test was used to test the significance of association between discrete variables, whereas student *t*-test was used to test the significance of differences between continuous variables. Statistically significance was considered when P

< 0.05. All data were analysed using Microsoft excel 2010 software.

Results

Age of both groups were compared there was no significant difference with P < 0.05 (27.9±0.27 for control, 27.6±0.28 for study group), gravidity and parity also were compared between both groups, the difference was statistically insignificant (gravidity 3.5±1.7 and parity 2.5±1.7 for control, for study group gravidity was 3.3±1.6 and parity was 2.2±1.6).

Table 1 showed the frequency of miscarriage and preterm deliveries among cases and controls interpreted as percentage, the frequency of miscarriage among those who breastfed their babies during pregnancy were significantly lower than among those who didn't breastfed during pregnancy (P < 0.05), however, there was no statistically significant association between breastfeeding during pregnancy and preterm deliveries.

Table 1. Frequency of miscarriage, preterm and term delivery among the studied groups

Group	Control group		Study group		P value
	N	%	N	%	
Miscarriage	29	10.35%	11	5.12%	0.0164
Preterm	12	4.29%	13	6.05%	0.4967
Full term	239	85.36%	191	88.83%	0.3136
Total	280	100%	215	100%	0.0801
Odds ratio	1.3652	95% CI	0.797 to 2.34		0.2571

Table 2 showed the difference in the frequencies of miscarriage among those who breastfed their babies exclusively during the current pregnancies compared to those in whom the breastfeeding were nonexclusive interpreted as percentages. There was no statistically significant association between the type of breastfeeding and the frequency of miscarriage with *P* > 0.05.

Table 2. Effect of type of breastfeeding on the frequency of miscarriage in lactating women

Group	Breastfeeding				*P* value
	Exclusive		Non-exclusive		
	N	%	N	%	
Miscarriage	6	8.11	5	5.34	0.7871
Total	99		116		

Table 3 compared the birth weight of neonates of mothers who were breastfeeding their previous child during pregnancy and those who weren't breastfeeding. There was insignificant difference in the mean birth weight of the neonates between the two groups.

Table 3. Illustrates the delivery birth weight of term neonates and breast fed mothers

Groups	Delivery weight		P value
	N	Mean ± SE	
Control group	239	3.197 ± 0.02	0.4312
Studied group	189	3.202 ± 0.02	

Discussion

Miscarriage and preterm deliveries are important problems of pregnancy [18,19]. In many developing countries, women get pregnant while breastfeeding their babies [20], and they may have concerns about their safety during current pregnancies and the impact on the new baby. There was significantly lower frequency of miscarriage among those who breastfed during pregnancy compared to those who didn't.

The frequency of preterm deliveries is higher among women who breastfed during pregnancy, however, this doesn't reach statistical significance. This result is in agreement with the result of Moscona's (1993) survey and the comparative study of Madarshahian [21].

Since breastfeeding stimulates the posterior pituitary gland to release oxytocin [22], so theoretically it increases the risk of preterm labours and deliveries, however, clinically this is not the case. The protective mechanism against miscarriage and preterm labour can be explained by "oxytocin receptor sites "theory: the uterine cells that detect the presence of oxytocin and cause a contraction are scarce until term, increasing gradually after that [23]. Also the absence of gap junction proteins before term renders the uterus relatively insensitive to oxytocin [24].

Other protective factor is possibly progesterone which stands between oxytocin and its receptor throughout pregnancy [25].

There was insignificant difference in the frequency of miscarriage among exclusive and non-exclusive breast feeders. This means that the frequency of suckling in exclusive breastfeeding doesn't increase the risk of miscarriage because the uterus in early pregnancy is irresponsive to increasing release of oxytocin as mentioned above [23 ,24].

There was statistically insignificant difference in neonatal birth weight among study and control groups. This is consistent with the result of Merchant *et al* study, which showed that overlapping breastfeeding and pregnancy was associated with a non-significant decrease of 57 gram in birth weight [26]; also it is consistent with the result of Madarshahian study [27].

This can be explained by "adaptive mechanism" that the women adopt during pregnancy in which the women use energy more efficiently despite high energetic burden of reproduction, this is proposed on the basis of good reproductive outcomes despite low measured levels of intake relative to the calculated required intake [28].

In conclusion, breastfeeding during early pregnancy doesn't increase the risk of miscarriage whether it is exclusive or non exclusive. Moreover, breastfeeding during pregnancy neither increases the risk of preterm deliveries nor affects neonatal birth weight.

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E-mail: jubranhassan@gmail.com
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Metabolic Risk Factors for Urolithiasis in a Group of Iraqi Children

Shatha H. Ali[1] *CABP*, **Fadhil Sh. Hussein**[2] *FICMS CABP*, **Faleeha O. Hasan**[3] *FICMS CABP*

[1]Dept. of Pediatrics, College of Medicine, Al-Nahrain University, [2]Al-Imamian Al-Kadhymain Medical City, [3]The Central Child Teaching Hospital, Baghdad, Iraq

Abstract

Background Pediatric urolithiasis (UL) should not be underestimated, because it is associated with significant morbidity, particularly because stones tend to recur.

Objective To study the demographic characteristics, clinical manifestations, metabolic disorders and some risk factors for stone formation in a group of Iraqi children.

Methods A total of 96 children with UL comprised 66 males and 33 females with an age range 0.1-14 years were studied for the period from 1st of January 2009 to the end of December 2011.

Results Positive family history was present in 29 patients (30.2%); all of them had metabolic disorder. Recurrence rate of stone was recorded in 41 (42.7%); of them 28 (68.3%) had metabolic disorder. The commonest clinical presentation was urinary tract infection in 40 (41.7%). Urine culture was positive in 57 (59.3%) children predominated by E.Coli in 23 (40.3%). Twenty four hour urine collection were positive for metabolic disorders in 84 patients (87.5%) and mainly hypercalciurea in 53 (63 %), hyperoxalurea in 31 (36.9%), hyperuricosurea in 23 (27.3%), and cystinurea in 15 (17.8%). Staghom calculi were detected in 6 patients (6.2%), all are associated with infection. Chemical analysis show calcium salt as major component in 22 out of 33 stones (66.6%). Predisposing risk factors for stone formation was established in 91 patients (94.8%) while no etiology could be found in 5 (5.2%). Metabolic disorders were the major risk for stone formation in 54 (56.3%), infection in 21(21.8%) and renal anomalies in 16 (16.7%).

Conclusion Metabolic disorders were found to be the major predisposing factors to stone formation among this group of Iraqi children. Early presentation, family history of stone disease, high recurrence rate of UL, bilateral and multiple stones are all indicators for metabolic disorders which mandate complete metabolic evaluation in pediatric stone formers.

Keywords Urolithiasis, stones, metabolic, children

Introduction

Pediatric Urolithiasis (UL) should not be underestimated, because it is associated with significant morbidity, particularly because stones tend to recur. As compared with the adult population, a far higher proportion of pediatric patients have a well-defined underlying condition that favors stone formation (e.g., metabolic disorders, infections, urinary tract anomalies). For these reasons, it is imperative to evaluate carefully all pediatric stone patients as soon as stone disease is recognized and to pay great attention to the prevention of further stone formation [1,2].

The two mechanisms by which metabolic factors enhance stone formation include: 1. Solute excess: high urinary concentrations of calcium, oxalate, uric acid, and cystine due to increased renal excretion and/or low urine volume cause solute excess. 2. Decrease levels of inhibitors of

stone formation: Natural inhibitors of urinary stone formation include citrate, magnesium, and pyrophosphate [3-7].

In 20 to 25 percent of children with UL, urinary tract infection (UTI) is detected or there is a history of a UTI. Infection may be the primary cause of a stone or occur concomitantly with a underlying urinary metabolic abnormality or structural abnormality [8].

Congenital and structural abnormalities that are accompanied by urinary stasis are associated with UL. Urinary stasis predisposes to crystal and stone formation [2,9]. Patients who have surgically augmented bladders are at risk for nephrolithiasis, most commonly bladder stones composed of struvite [10].

The incidence of UL in children varies worldwide with the highest incidence occurring in endemic areas, such as in Turkey and Thailand. Stones were more commonly found in Caucasian children and rarely in African-American children. Incidence of UL is lower in children than in adults [4]. The Objectives of this study were to evaluate pediatric UL in a group of Iraqi children regarding some demographic characteristics, clinical presentation, laboratory findings, metabolic disorders and characteristics of stones and chemical composition.

Methods

This cross sectional study was based on 96 children with UL for the period from 1st of January 2009 to the end of December 2011. Those patients with UL were evaluated, treated, and followed up in the pediatric nephrology clinic in Al-Imamian Al-Kadhymian Medical City in Baghdad.

Presence of stone disease was confirmed in all case; radiologically by renal sonography with or without plain abdominal radiograph or patient passed at least one urinary calculus.

All children with renal stones whether newly diagnosed or recurrence were included. Children with renal tubular acidosis or nephrocalcinosis were excluded from the study.

Full recording of the patient characteristics and stone data was done. All children were examined physically and underwent the following investigations: Urinalysis, urine culture. Blood biochemistry test: urea, creatinine, sodium, potassium, calcium, phosphorus, uric acid, and alkaline phosphatase level. Twenty-four hour urine determination of urinary calcium, oxalate and uric acid was performed for all children.

Hypercalciuria (HCa) was defined as urine calcium excretion >4 mg/kg/24 h, Hyperoxaluria (HOx) was defined as urine oxalate excretion >55 mg/1.73 m^2/24 h, Hyperuricosuria (HUr) was defined as uric acid excretion > 815 mg/1.73 m^2/24 h [3,4,11-17].

Serum and urine amino acid excretion were tested for all children using paper chromatography and the nitroprusside test for diagnosis of cystinuria [3,13,17].

Stones from 33 patients that were removed surgically or obtained by spontaneous passage were analyzed chemically.

Voiding cystourethrography (VCUG) and intravenous pyelography (IVP) were done to some patients as indicated. VCUG was done in patients with recurrent urinary tract infection (UTI), and suspected vesicoureteral reflux (VUR). IVP was performed in some cases of suspected renal anomalies.

All of the urinary stones were classified into four groups according to the predisposing risk factor for their occurrence [4,13,15-18].

(1) Metabolic stones: stones predisposed by metabolic disorder.

(2) Anatomic stones: stones that formed with anomalies of urinary system.

(3) Infection stones: children with recurrent UTI with one or more of the following: **A**. staghorn calculi **B**. urine culture of Proteus **C**. Stone chemical analysis of calcium phosphate carbonate, or magnesium ammonium phosphate **D**. associated VUR

(4) Idiopathic stones: The stones that are formed without metabolic, anatomic or infectious etiology.

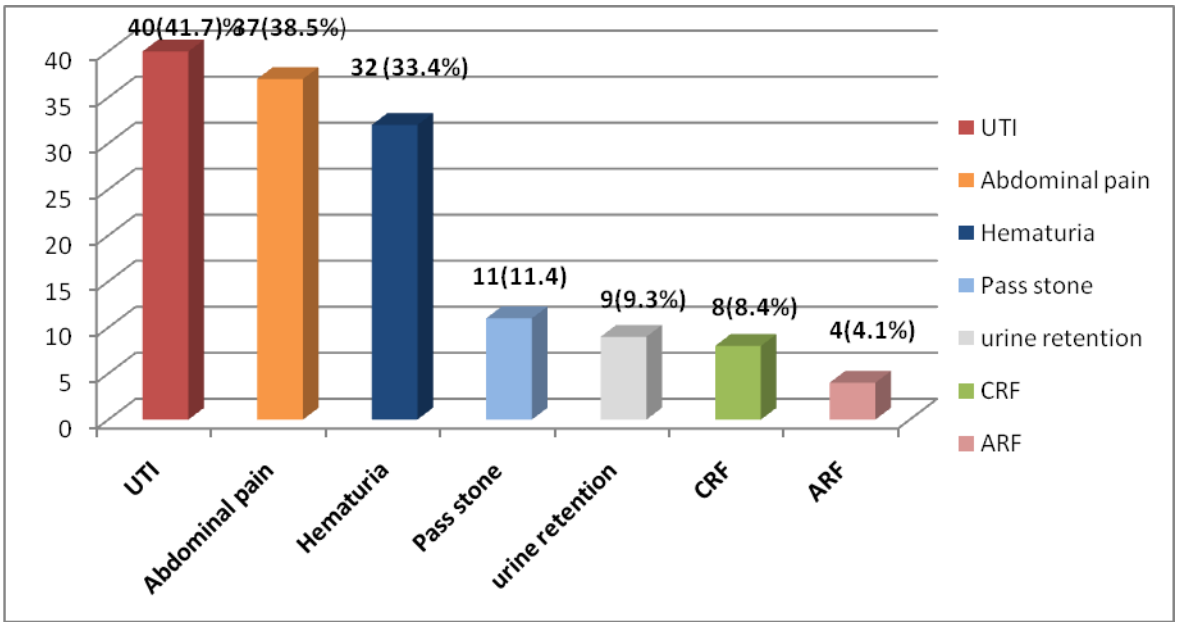
Patients received follow-up testing every 1-2 months; serial US was used to track UL status and was scheduled at every 4-6 months.

Results

Patient population consisted of 96 children with UL, 63 (65.6%) were males and 33 (34.3%) were females with male to female ratio 1.9:1. Their age ranged between 0.1 to 14 years (mean age 3.54 ± 3.389 SD years). The majority of patients 46 (47.9%) were in the 1-5 years age group.

Family history of UL was reported in 29 patients (30.2%); all of them had metabolic disorders. Forty-one patients (42.7%) experienced recurrences, and 28 patients of them (68.3%) had metabolic disorders. Recurrence occurred after an initial diagnosis of stone disease during their lifetime.

Clinical presentation was dominated by UTI in 40 (41.7%) as seen in Fig. 1. Urine culture was positive in more than half of children 57 (59.3%) which is predominated by *E. coli* in 23 (40.3%) of positive cases (Fig. 2).



*Patient may have more than one presentation, CRF: Chronic Renal Failure, ARF: Acute Renal Failure

Fig. 1. Clinical presentation of patients with Urolithiasis

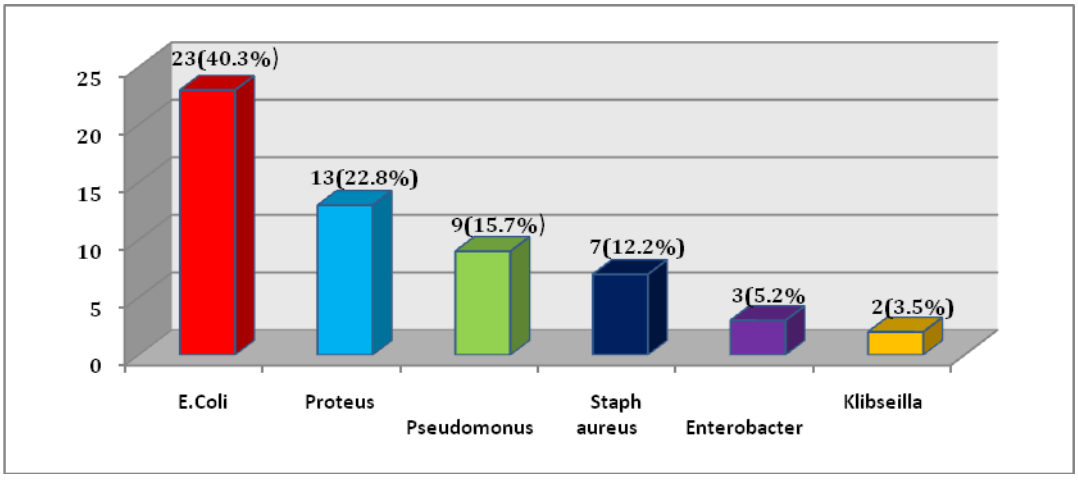


Fig. 2. Urine Culture results of patients with Urolithiasis

Twenty-four hours urine results were positive for metabolic disorders in most cases 84 (87.5%). Hypercalciurea was the commonest detected in 53 patients (63%), 46 out of 84 patients with metabolic disorder had single metabolic urine disorder while the other 38 had multiple urine metabolic disorders (Table 1).

Table 1. Single and multiple urine metabolic disorder in patients with urolithiasis

Single urine metabolic disorder in 46 patients with UL		
Disorder	**No.**	**%**
Hypercalciurea	17	36.9
Hperuricosurea	18	39.1
Cystinurea	9	19.6
Hyperoxalurea	2	4.4
Multiple urine metabolic disorder in 38 patients with UL		
Disorder	**No.**	**%**
Hypercalciurea-hperoxalurea	29	76.3
Hypercalciurea-cystinurea	4	10.5
Hypercalciurea-hyperuricosurea	3	7.9
Hyperuricosurea-cystinurea	2	5.3

Stone data reflect that 36 patients (37.5%) had multiple stones, 26 (72.2%) of the multiple calculi were related to metabolic disorders. Most patients 40 (41.6%) had small size stones (<1 cm) as calculated by US, Staghrn calculi were detected in 6 patients (6.2%), all were associated with infection (Table 2).

Forty-five patients (46.8%) had stones in more than one site. Right kidney was the commonest site for stone location, involved in 58 patients (60.4%), while urinary bladder was involved in 6 patients (6.2%). Bilateral stones found in 41 (42.7%) of whom 23 (56%) had metabolic disorders, and 5 out of 6 bladder stones were proved as infection stone. Chemical stone analysis was done for 33 patients as shown in Table 2. Calcium oxalates is the most common mixture identified in 13(39.4%) patients as pure or mixed.

Risk factors for stone formation were established in 91 (94.8%) while no predisposing factor could be found in 5 (5.2%). Metabolic disorders were the major risk for stone formation in 54(56.3%), infection in 21 (21.8%) and renal anomalies in 16 (16.7%) as shown in Fig. 3.

Table 2. Stone Data in patients with urolithisis

Feature		No	%
No. of stones	1 stone	33	34.3
	2 stones	27	28.1
	>2 stones	36	37.5
Size of stones	<1cm	40	41.6
	1-2cm	32	33.3
	>2cm	18	18.7
	Staghorn	6	6.2
Composition of stones	Ca+Oxalate	8	24.2
	Ca+Phosphate (ph)	3	9
	Ca+UA	4	12.1
	Ca+UA+Ph+Mg	1	3
	Ca+UA+Ph+oxalate	1	3
	Ca+Oxalate+UA	2	6
	Uric Acid	2	9
	UA+Ammonium +Ph	1	3
	Ca+oxalate+carbonate	2	6
	Cystine	7	21.2
	Ca+Cystine+Ph	1	3
	UA+Ph+Carbonate	1	3

As described from above results, metabolic disorders were detected in 84 patients; as a pure metabolic disorder in 54 patients (56.3%), and in other 30 as mixed metabolic disorder with other factor.

Although UTI was documented in 57patients at presentation, only 21 out of 57 of those patients (21.8%) fulfill the criteria of infection stones; from those 21 patients; 16 patients were associated with metabolic disorders, one patient with anomalies and 4 patients had metabolic infectious and anomalies.

Sixteen (16.7%) patients with UL were due to anatomical renal anomalies; 10 out of these 16 were associated with metabolic disorders and the other 6 patients had pure renal anomalies; the associated renal anomalies were described in (Table 3).

Discussion

Many studies have reported a male predominance in childhood UL [4,14,15,18-20]. In agreement with many studies [14,15,18,19,21-23], nearly half of our patients were below 5 years of age. Early evaluation by ultrasonography in addition to high rate of metabolic disorders among our cases, which led to early presentation, was a leading cause of early identification of stone disease

A family history of UL was reported in wide range of 7.3-78.7% in previous studies [3,4,14,15,18,19, 22-24] .In this study, all patients with family history of UL had metabolic disorder for UL. This finding was highlighted by 3 previous studies [4,15,18]. This finding reflects the genetic basis of metabolic disorders for stone formation.

Recurrence risk in this study was 42.7% and 68% of them had metabolic disorders. Recent Turkish study found nearly similar rate of recurrence (44%) and that children with at least one identifiable metabolic abnormality tended to have higher recurrence rates than the others [25]. Recurrence of UL is a consequence of most metabolic disorders [8].

Most common reported clinical presentation of UL in children are abdominal pain and hematuria [3-5,14,15,18,19,23,24,26]. Not so like our results, which showed predominance of UTI (41.7%)? Several studies have noted a strong association between UL and UTI [3,5,14,18,19,21-24,27]. We believe that UTI was a complication of stones rather than predisposing factor, as infection stones were detected in only 21 patients out from 57 patients with documented UTI among our cases.

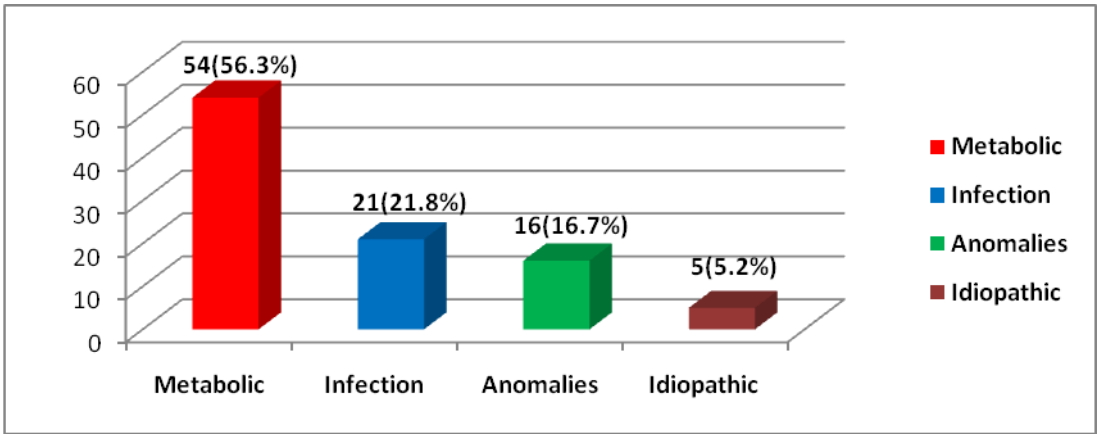


Fig. 3. Predisposing risk factors of patients with urolithiasis

Similar to Al Rasheed et al study [28], *Escherichia coli* was the most frequent bacteria isolated while Proteus dominated in others [18,23].

Metabolic disorders were detected with variable rates in children with UL ranging from 10.6% to 92% from different regions [3-5,14,15,18,28].

In a previous Iraqi series, metabolic disorders were detected in 72% of children with UL [15], compared with 87.5% in this study. This increase in metabolic disorders among Iraqi children with UL reflects changing environment and dietary habits both as important risk factors for UL, which need to be further studied.

In agreement with most studies from different regions [3-5,15,18,19,23,27], we detected hyper-calciuria as commonest metabolic disorder.

Interestingly, cystinuria was detected in 15 of our patients (17.8%). Lower results came from other studies [4,14,18,19,23,24,27]. Our cultural habits with high rate of first-degree cousin marriages might explain our results as cystinuria is known autosomal recessive inherited disorder.

Multiple and bilateral stones were recorded in variable rates in many studies [4,14,18,19,24,28]. In this study, 72.2% of the multiple stones and 67.6% of the bilateral stones were related to metabolic disorders. This relation between

metabolic disorder with bilateral and multiple stones was confirmed by 2 authors [4,31]. These findings led us to think that metabolic disorders dominate cases with multiple and bilateral stone. A correlation between staghorn calculi and infective stones was noted in this study, also found by many authors [17,29,30]. In consistent with results of many studies [3,4,14,15,18,19,24,25,28], majority of stones were located in the upper urinary tract among our patients.

Table 3. Associated renal anomalies in 16 patients with urolithiasis

Associated anomalies/causes	No.	%
Neurogenic bladder	6	6.2
Single kidney	3	3.1
Polycystic kidney	3	3.1
PUJ obstruction	2	2.08
Duplex system	2	2.08

In agreement with several studies, calcium oxalate was the most frequent chemical compound in UL [4,14,18,19,22,24,25,26,28]. Calcium oxalate stones are linked to dietary habits, although this effect is more prominent among adults more than children However, Calcium oxalate stone caused by genetic diseases is proportionately more frequent in children [18].

Similar to us, most literature reviews from various regions of the world revealed an underlying predisposing factor for stone formation in a large proportion of their studied series. (3, 4, 14, 15, 19, 23, 24, 26, 27).

Like our results, metabolic disorders ranked first etiology for UL in most studies [3,4,14,15,19,23,24,26,27]. Over the past decades, the etiology of UL in children has shifted from predominantly infectious to metabolic causes [14,32]. Early presentation, family history of stone disease, high recurrence rate of UL, bilateral and multiple stones are all indicators for metabolic disorders, which were observed in this study, and this observation was also confirmed by a recent study from Turkey [4]. Most of the studies that addressed etiologic classification reported a ratio of 10.7–26% for Infectious stones [4,18,24,26,28] which is near to our results.

Previous studies reported similar renal anomalies with various rates [4,18,19,26,28]. Urinary tract anomalies with stasis, but no infection, leading to CaOx stones, while the growth of calcium phosphate stones is facilitated by infection and a high urinary pH [18,26]. The profile of predisposing risk factors for UL of the present study was in accordance with previous Iraqi study held on 2005 [15].

In conclusion, UL among a group of Iraqi children had male predominance, early onset of presentation, high rate of positive family history and recurrence of stone disease. UTI was the commonest clinical presentation with *E. coli* as predominant pathogen isolated by urine culture. Hypercalciuria was the commonest metabolic abnormality as single or multiple and Calcium oxalate was the commonest type of stones. Predisposing factor of UL was established in majority of cases.

Early presentation, family history of stone disease, high recurrence rate of UL, bilateral and multiple stones are all indicators for metabolic disorders, which mandate complete metabolic evaluation in pediatric stone formers to determine the possible metabolic disorder with early treatment.

Future studies are needed including patients of UL from other Iraqi governorates. Pediatrician should have higher concern of possible UL with mentioned clinical presentation, with early referral to pediatric nephrology / Urology clinic.

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Correspondence to Dr. Shatha H. Ali
P. O. Box 70074, Baghdad – Iraq
Mobile: + 964 7901479929
E-mail: shathah666@yahoo.com

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Immunohistocemichal Assessment of the Role of WT1 Protein Expression in CML and its Correlation with CD 31 as an Angiogenic Marker

Mustpha A. Mukeef[1] *MSc*, **Raad J. Musa**[2] *FICMS,* **Qais A. Al-Oqaily**[2] *MSc,* **Aliaa M. Saeed**[3] *MBChB*

[1]Laboratories of Al-Imamian Al-Kadmyian Medical City, [2]Dept. of Pathology & Forensic Medicine, College of Medicine, Al-Nahrain University [3]Central Health Laboratories, Ministry of Health

Abstract

Background Several studies have demonstrated that Wilms' tumour gene 1 (WT1) is consistently overexpressed in most forms of leukemias, and the usefulness of quantitative assessment of WT1 expression as a molecular marker for minimal residual disease (MRD). Many suggest a role of WT1 for angiogenesis in hematological malignancies, WT1 is also expressed in a large variety of tumour blood vessels, and some suggests that it might be a general marker for angiogenesis.

Objective To assess the role of WT1 protein expression immunohistochemically in chronic myeloid leukemia (CML) and to determine whether there is a correlation between WT1 protein expression and CD31 expression as a marker of angiogenesis.

Methods This study involved 16 cases of newly diagnosed CML. In addition, 20 age matched control cases were involved having no apparent bone marrow pathology. Immunohistochemistry was done on bone marrow biopsies using Anti-WT1 and Anti-CD31 Monoclonal antibodies.

Results There was a significant increase in WT1 protein expression in CML cases, as well as an increase in CD31 expression; however, there was no significant correlation between WT1 expression and hematological parameters (WBC count, platelets count, PCV level, and peripheral blood blast %) and CD31 expression.

Conclusion This study showed that WT1 is overexpressed in CML patients, while it was undetected in controls, thus we may propose that it maybe used as an auxiliary marker for the disease. *WT1* expression was not found to be of prognostic significance. Moreover CD31 as a marker for angiogenesis was significantly increased in CML but did not correlate with WT1 expression.

Key words WT1, chronic myeloid leukemia, immunohistochemistry

Introduction

The WT1 gene, located on chromosome 11p13, was first identified in patients with Wilms tumor; it encodes a transcription factor involved in normal and malignant hematopoiesis, unlike other tumor suppressor genes, such as Rb and p53, the expression of the WT1 gene is restricted to a limited set of tissues (fetal kidney, ovary, testis, and spleen) [(1)]. More recently, *WT1* overexpression was detected in several haematological and solid malignancies. Additional studies revealed that it had a role in the initiation phase of the malignant diseases [(2)]. Since WT1 is believed to be relevant in the maintenance of the malignant phenotype of the tumour cells and is mostly restricted to malignant tissues, it is an attractive target for immunotherapy [(2)].

Chronic myeloid leukemia (CML) is a malignant clonal blood disease that originates from a pluripotent hematopoietic stem cell. The

cytogenetic hallmark of CML, the Philadelphia chromosome (Ph), is formed as a result of reciprocal translocation between chromosomes 9 and 22, leading to the uncontrolled proliferation of the bone marrow cells [3].

Angiogenesis is the formation of new blood vessels from pre-existing vessels during adult life [4]. Many studies suggest a role for angiogenesis not only in the pathogenesis of solid tumors but also in hematological malignancies like acute and chronic leukemia, lymphoma, myelodysplastic syndromes, myeloproliferative neoplasms, and multiple myeloma [5]; furthermore, WT1 is also expressed in a large variety of tumour blood vessels [6], as it is involved in endothelial cell proliferation, vascular formation and migration, indicating that it might be a general marker for angiogenesis [2].

Since WT1 is a marker of angiogenesis and it is believed to be relevant in the maintenance of the malignant phenotype of the tumour cells [2], this study will assess the expression of WT1 in chronic myeloid leukemia, and investigate if there is a correlation between the WT1 expression and angiogenesis (as marked by CD31 expression) in CML which might help for future therapeutic trials.

Methods

Patients and sampling

This cross sectional case control study was conducted from March 2011 to July 2012 on the trephine biopsy of 16 newly diagnosed CML patients including 10 in chronic, 3 in blastic, and 3 in accelerated phases. In addition to 20 age matched control cases with benign reactive marrow with no evidence of hematological malignancy, the cases were collected from The Hematology Ward of Baghdad Teaching Hospital. This study was ethically approved by the Ministry of Health. Clinical and laboratory information regarding age, sex, packed cell volume (PCV), white blood cell (WBC) count, platelets count, blasts percent, were obtained directly from the patient through taking history and examination at time of diagnosis during the clinical course and before taking chemotherapy.

From each formalin fixed paraffin embedded bone marrow biopsy used in this study, 3 sections of 4 µm thick were taken; one representative section was stained with Hematoxylin and Eosin (H&E) stain and was reviewed, while the other sections were stained immunohistochemically with WT1 and CD31 monoclonal antibodies respectively.

Immunhistochemistry

The primary antibodies used in this study were: monoclonal mouse anti-human Wilms' Tumor 1 (WT1) protein, clone 6F-H2 (Dako Cytomation), prediluted monoclonal mouse antiendothelial cell marker (CD31) antibody, clone JC70A (Dako Cytomation); while the immunohistochemistry (IHC) secondary detection kit used was immunoperoxidase secondary detection kit (DakoCytomation IHC kit LSAB2 System-HRP, code K0679) which was purchased from DAKO, Denmark. The immunohistochemical staining procedure was done according to the manufacturer's instructions. Positive staining is expressed as a brown color, in which brown cytoplasmic staining of endothelial cells is considered positive reaction for CD31 [7]; and staining of either the nucleus and/or the cytoplasm indicated a positive result for WT1 [8].

For IHC technical quality control: tonsils tissue which was taken from a healthy young patient who had no other known disease other than inflamed tonsils that required tonsillectomy were used as a positive control tissue for CD31, while Wilms Tumor tissues were used as a positive control tissue for WT1 staining. Technical negative control was performed by omission of the primary antibody.

Scoring of immunohistochemical staining

Scoring of immunohistochemical staining was performed using specialized automated cellular image analysis system, Digimizer software, version 3.7.0, that allows precise manual measurements as well as automatic object detection with measurements of object characteristics (Fig. 1) [9].

For purpose of statistical analysis, the following variables were used:

A. Color Intensity: the average intensity of the brown color for the selected objects depending on the expression of antigens in the cells.

B. Fractional area stained: which equals to *[(mean area* Number of objects)/area of a single image field]*100*

C. Digital Labeling Index: for better estimation of the immunohistochemical expression of the WT1, CD31, we used an arithmetic tool named as Digital Labeling Index. This tool is calculated according to the following formula: (Fractional area * reverse Intensity). This parameter combines both the Fractional area and the Intensity [9].

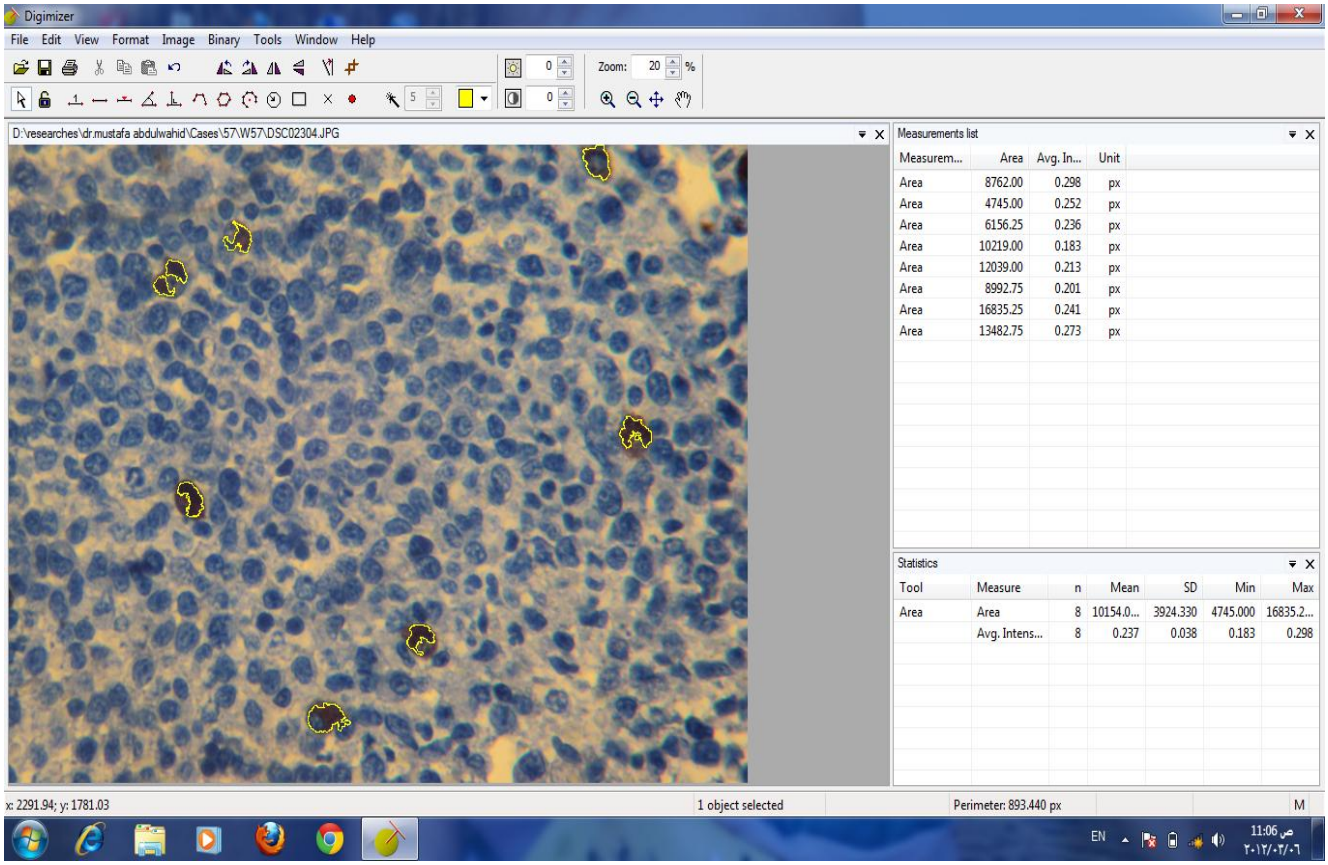

Fig. 1. Image analysis in Digimizer software

Statistical Analysis

Data were analyzed using SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as mean± SEM, frequency was used to express discrete data. ANOVA was used to analyze numeric data while Chi-square was used to analyze discrete data, and benferroni test was used for multiple comparisons. Spearman rank correlation was used to determine relation between various markers. *P* Value of < 0.05 was considered significant.

Results

This study included 16 cases of CML, divided into 10 cases in chronic phase, 3 cases in accelerated phase, and 3 cases in blastic phase, in addition to 20 control cases; in CML. Eleven of the patients were males (69%) and 5 of them were females (31%) with a male to female ratio of 2.2:1; on the other hand, 12 of 20 control persons were males (60%), while 9 were females (40%) with a male to female ratio of 1.3:1.The age range of patients with CML was between 32-57 years with a mean of 46.5±7.4 years, by dividing the patients according to 10 year intervals, the largest number of patients (7) fall

in the age group 40-49. Regarding the comtrol group, the age range was between 24-71 years and the mean was 49.60±14.17 years, with the largest number of cases falling in the 50-59 years age group.

Regarding Immunohistochemical staining results, we depended on Digital Labelling Index (DLI) parameters in considering what is positive and what is negative; 11 out of the 16 CML cases were positive for WT1 DLI, while none of the control cases were positive; regarding CD31, 15 cases were positive in CML, while none of the control cases were positive. WT1 DLI was significantly higher in CML than controls 4.17±3.67 (P = 0.003).

WT1 DLI was significantly higher in blastic and accelerated phases of CML than Chronic phase (6.46±4.34, 9.49±1.47 respectively versus 2.27±2.81 in chronic phase, P = 0.032 and 0.001 respectively) as seen in Table 1.

Table 1: Comparison of WT1 DLI between CML phases Subclasses

Parameter	CML phase	Mean (DLI)±SD	*Vs*	CML phase	Mean±SD	P	SE
WT1	CML chronic	2.27±2.81	*Vs*	CML blastic	6.46±4.34	0.032	1.53
				CML accelerated	9.49±1.49	0.001	1.69
	CML blastic	6.46±4.34	*Vs*	CML accelerated	8.49±2.49	0.154	1.94

WT1 DLI was not significantly correlated with age and gender of the patient. WT1 DLI was significantly positively correlated with blast % BMA in CML (r = 0.619, p = 0.011), while it was not significantly correlated with PCV, WBC count or Platelets count; on the other hand. Angiogenesis parameter used in this study, CD31 DLI, was significantly higher in CML than in controls (8.38±2.51 versus 0.1±0.01, P = 0.004). There was no significant correlation between WT1 DLI with CD31 DLI in CML.

Discussion

The concrete role of WT1 in hematopoiesis and leukemogenesis remains unclear. Studies on the oncogenic activity of WT1 have led to conflicting results demonstrating cell proliferation in some and cell growth arrest in others [10]. In the presented cross sectional case control study, WT1 DLI was positive in 11 CML cases (68.75%) while none of the control cases were positive for WT1. These results were in accordance to other studies such as Rosenfeld et al [11], who found, using Real Time PCR technique, that WT1 gene was overexpressed in all cases of CML; while Huang et al [12] found, using conventional nested PCR not QR-PCR, that 17 out of 37 CML showed WT1 expression.

Interestingly, we have found that WT1 protein expression level was significantly higher in CML accelerated and CML blastic phases than CML chronic phase, while there was no significant difference in WT1 expression level between CML accelerated and CML blastic phases. This goes with Kreuzer et al study [13], which showed WT1 overexpression, using Real Time- PCR, in all CML patients studied, but revealed differences in WT1 expression levels within this patient population; similarly Huang et al [12] showed that 5/18 (27.7%) CML blastic crisis patients, 1/5 (20%) CML patients in accelerated phase, and 1/10 (10%) CML patients in chronic phase have had high WT1 expression level; on the other hand, using conventional PCR, Menssen [14] revealed overexpression of WT1 in all blast crisis cases but not in chronic phase cases. These data support the notion that increased levels of *WT1* expression are indeed specific to leukemic blasts with respect to normal hematopoietic progenitors and not a simple consequence of the differentiation degree.

In this study, WT1 protein expression in CML was not significantly associated with gender and age of the patients, and WT1 protein expression was not significantly associated with various hematological parameters (WBC count, platelets count, PCV level, and peripheral blood blast %)

which goes with Sadek et al [1], Karakas et al [15], Gu Wy et al [16]; on the other hand, it was positively correlated with Blasts % in bone marrow aspirate. Interestingly, Cao et al [17] found that WT1 expression levels in CML patients in accelerate phase or blast crisis were strikingly higher than those in non-leukemic patients or CML patients in chronic phase; thus, it appears that WT1 gene expression is associated with immature cells from which leukemic cells in CML originate.

In CML, CD31 DLI was significantly higher than in controls; which also goes with Alvaro et al [18] and Hans et al [19], which have found a significant increase in angiogenesis in CML compared with healthy control cases.

In this study, there was no significant correlation between WT1 protein expression and CD31 in CML, this does not go in line with Wagner et al [6], who found that WT1 might be involved in tumour angiogenesis, in which endothelial WT1 expression was detected in 95% of 113 AML cases of different origin and that transcriptional activation of ETS-1 by the Wilms' tumour suppressor WT1 is a crucial step in tumour vascularization via regulation of endothelial cell proliferation and migration; moreover Trka et al [20], have suggested that WT1expression can be stimulated by hypoxia, which involves activation of the WT1 promoter by HIF-1. The discrepancy between our finding and other studies may be due to the fact that others have used more sensitive methods (PCR) for evaluating WT1 than Immunohistochemical staining procedure we used in addition to the smaller sample size.

There are several controversies surrounding reported data on the prognostic significance of WT1 expression, which is mainly because of the limited number of patients and the diversity of methods used; while some groups have shown that high levels of WT1 coincide with worse prognosis [21-24], suggesting that WT1 levels could be useful for predicting prognosis in such patients, no evidence was found that the level of WT1 at diagnosis was an independent prognostic factor for survival, just as some studies failed to show any correlation between initial WT1 levels and outcome of the disease at all [25,26]. These discrepancies may be due to differing methodologies, for example, real-time PCR versus end-point analysis or due to patient selection. Moreover, based on results similar to those found above, it is strongly believed that WT1 can become a target for immunotherapeutic approaches as suggested by Rosenfeld et al [11], upcoming data support this hypothesis, as sera from many AML, CML, and MDS patients have anti-WT1 antibodies [11].

In conclusion, this study showed that WT1 was overexpressed in 68.75% of CML patients; taken together with longitudinal analyses of WT1 expression in healthy donors, which was undetectable. CD 31 expression (as a marker of angiogenesis) was significantly higher in CML in comparison with control cases but there was no significant correlation between its expression and WT1 expression.

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Correspondence to Dr. Mustpha A. Mukeef
E-Mail: drmustafa78@yahoo.com
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