Flow Cytometry of Breast Cancer Resistant Protein and microRNA in Breast Cancer Patients Post Metformin Effect

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Abstract

The goal of the present study is to investigate the role of metformin (MF) as a target of miRNAs in breast cancer resistant protein (BCRP) inhibition in an attempt to develop treatment strategies that may improve the response of breast cancer (BC) patients to chemotherapy (CT). In order to fulfill this target, non-diabetic female subjects were categorized into three groups: control group (group 1) (n=5), CT group of BC patients (group 2) (n=25) and CT plus MF group of BC patients (group 3) (n=25). All patients were subjected to full history taking, laboratory studies including mammogram, chest X-ray, pelvic-abdominal ultrasound and isotopic bone scan, in addition to ER and PR states. CT group was treated with neoadjuvent CT in the form of 5-FU (500 mg/m^2), Adriamycin (50 mg/m^2) and cyclophosphamide (500 mg/m^2). Flow cytometry (FC) of BCRP and MiRNA was carried out on blood samples at every cycle of treatment for all partners.

The results showed the presence of miRNA was higher than the presence of BCRP in the normal healthy control group. In most cases of CT and CT plus MF groups (group 2, 3) it was well noticed that the amount of BCRP in the blood samples exceeded that of miRNA illustrated the dysregulation of miRNA in BC patients and also to prove the basic role of BCRP as a multidrug resistance (MDR) for chemotherapeutic agents in patients with BC.

It is concluded that the role of MF was well proved in targeting of miRNA to reinforce BC medication, so oncologists can be advised to use MF equivalent to CT in the recommended doses.

Keywords: Breast cancer, Breast cancer resistant protein, Chemotherapy, Flow cytometry, Metformin, Multidrug resistance, microRNAs.

Introduction

Breast cancer is the most commonly diagnosed cancer and worldwide it is considered the leading cause of cancer death in females, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths (Anderson et al., 2008).

Approximately half of the BC cases and 60% of the deaths are estimated to take place in developing countries, In Egypt, the median age at diagnosis for BC is ten years younger than in the United States and Europe (Omar et al., 2003).Cancer in young is generally more aggressive and results in lower survival rates, making early detection even more crucial and emphasizing the importance to raise BC awareness among young females (Sambanje et al., 2012).

Chemotherapy (CT) plays a key role in the treatment of BC; however, the widespread resistance to CT often results in the treatment failure. Drug resistance to chemotherapeutic agents accounts for treatment failure in more than 90% of patients with metastatic cancer.

Metformin (MF), the most common first-line drug in the treatment of type-2 diabetes, has been shown in previous studies to reduce BC risk, improve survival, and increase the effectiveness of CT.

The discovery of miRNAs has opened new avenues for BC metastasis research. Several lines of evidence now link miRNA dysregulation to carcinogenesis, progression, chemoresistance and recurrence in several cancers. Many recent studies on BC have clearly demonstrated that miRNAs can play an important role in the multistep process of metastasis, functioning as both activators and suppressors of metastasis by critically regulating various stages of migration and invasion. In BC, the expression levels of several miRNAs are significantly different between normal and cancerous tissues and between BCs of different molecular subtypes with a different prognosis. This study aimed to investigate the role of MF as a target of miRNAs in BCRP inhibition in an attempt to develop treatment strategies that may improve the response of breast cancer patients to chemotherapy.

Methods
Subjects

This study included 55 non-diabetic females aged from 37-70 years old, after written informed consent. Fifty patients were selected with histological proved BC patients admitted to Cancer Research and Management Department, Medical Research Institute, Alexandria University. Five volunteers of apparently normal breast and with comparable characteristics served as control group. Patients were subjected to:

Full history taking thorough clinical examination, laboratory studies including complete blood count, kidney and liver function tests, tumor markers (CEA and CA15.3), radiological examination including mammogram, chest X-ray, pelvic-abdominal ultrasound, isotopic bone scan and estrogen and progesterone receptors states.

Study design and treatment

Non-diabetic female subjects were categorized in three groups: control group (group1) (n=5), CT group of BC patients (group 2) (n=5) and CT plus MF group of BC patients (group 3) (n=25). Before every cycle of treatment, blood samples from all participants were used to determine the following parameters:

(1) Preparation of blood cells

Blood was collected from patients in the different groups and blood films were left to air dry. The slides were flood with Leishman's stain solution, wait for 8 minutes and then flood off with distilled water in 2 – 3 seconds.

(2) Flow cytometry of BCRP and miRNAs.

In summary, three tubes were taken, the first was for the negative control; the second was for anti-DDX6/RCK for miRNA and the third was for anti-BCRP.

Red blood corpuscles and platelets were lysed using lysing agent to see the antibodies (Abs) on the white blood corpuscles. 50μ of blood were taken and left for 20 minutes. The pellet was formed by vortex centrifuge for 3000 RPM. Wash by PBS after incubation for 30 minutes. The acquisition step was carried out to stop the reaction. The tubes were inserted to 10,000 events cytometer to get the final result sheet, that recorded one negative example and another positive example of the samples required. This was expressed by a dot plot.

Results
Patients' characteristics
A) Leishman's staining

In normal healthy controls, the basic blood cells are found clearly; In the CT group all blood cells were almost severely damaged. WBCs are burst and RBCs are overlapping at 1st cycle, 2nd cycle, and 3rd cycle of treatment. As for the MF group notable improvements were seen in which the neutrophils were retained with MF treatment and the biconcave shape of RBCs was greatly maintained gradually at 1st cycle 2nd cycle and 3rd cycle.

B) Flow cytometric analysis of BCRP and MicroRNA

The current study clarified that the values of BCRP at the third dose of (CT + MF) group was still higher than normal group but these values were less than that of (CT) group. This revealed the basic role of BCRP as multi-drug resistant although MF succeeded, to some extent in reducing these values (Table 1).

Table 2 displayed that MF at the third dose of treatment regained the normal value for miRNA (mean ± SD: 49.92 and median: 49.47), A statistical significant conclusion was recorded for the third group (CT + MF) exhibiting that MF had its role in reducing miRNA. This decrease in miRNA in turn represented the refinements in this group as a result of MF targeting miRNA.

Statistically in table 3, it had been shown that correlations existed between BCRP and MiRNA expressions and patients' outcome especially after MF medication.

Table 1. Comparison between the studied groups according to BCRP

	Control group (n=5)	CT group (doses)			MF group (doses)			F	p
		1st (n=25)	2nd (n=25)	3rd (n=25)	1st (n=25)	2nd (n=25)	3rd (n=25)		
BCRP % Min. – Max.	1.05 – 50.34	66.22 – 98.13	66.17 – 98.12	66.13 – 98.04	52.53 ± 88.10	44.98 – 77.05	44.15 – 59.26	71.327^*	$<0.001^*$
Mean ± SD.	26.29 ± 2.55	83.59 ± 9.74	83.46 ± 9.68	83.03 ± 9.68	63.54 ± 6.93	60.59 ± 8.35	51.87 ± 3.24		
Median	27.18	85.65	85.60	85.59	66.13	60.86	51.16		
p_1		$<0.001^*$	$<0.001^*$	$<0.001^*$	$<0.001^*$	$<0.001^*$	$<0.001^*$		
p_2					$<0.001^*$	0.866	$<0.001^*$		

F: F test (ANOVA)
p_1: p value for Post Hoc test (LSD) for comparing between control and each other group
p_2: p value for Post Hoc test (LSD) for comparing between CT group 1st with MF group 1st, CT group 2nd with MF group 2nd and CT group 3rd with MF group 3rd
*: Statistically significant at $p \leq 0.05$

Table 2. Comparison between the studied groups according to MiRNA

	Control group (n=5)	CT group (doses)			MF group (doses)			F	p
		1st (n=25)	2nd (n=25)	3rd (n=25)	1st (n=25)	2nd (n=25)	3rd (n=25)		
MiRNA %									
Min. – Max.	43.56 – 55.96	59.27 – 93.42	59.20 – 93.40	59.0 - 93.14	40.22 – 64.76	40.90 – 62.17	43.16 – 59.72	51.065[*]	<0.001[*]
Mean ± SD.	49.36 ± 5.59	76.69 ± 10.13	76.58 ± 10.12	76.21 ± 10.21	55.37 ± 7.99	54.05 ± 6.66	49.92 ± 4.62		
Median	49.0	77.09	76.92	76.61	59.24	55.43	49.47		
p₁		<0.001[*]	<0.001[*]	<0.001[*]	0.150	0.261	0.893		
p₂					<0.001[*]	<0.001[*]	<0.001[*]		

F: F test (ANOVA)
p_1 : p value for Post Hoc test (LSD) for comparing between control and each other group
p_2 : p value for Post Hoc test (LSD) for comparing between CT group II 1st with MF group III 1st , CT group II 2nd with MF group III 2nd and CT group II 3rd with MF group III 3rd
*: Statistically significant at p ≤ 0.05

Table 3. Correlation between BCRP with MiRNA in each studied group

BCRP	MiRNA	
	r	p
Control	0.128	0.838
Group II 1st dose CT	0.508[*]	0.010
Group II 2nd dose CT	0.514[*]	0.009
Group II 3rd dose CT	0.517[*]	0.008
Group III 1st dose CT + MF	0.644[*]	0.001
Group III 2nd dose CT + MF	0.328	0.109
Group III 3rd dose CT + MF	0.249	0.231

r: Pearson coefficient
*: Statistically significant at p ≤ 0.05

C) Flow cytometric analysis of results by dot plot
1. Flow cytometric results of BCRP
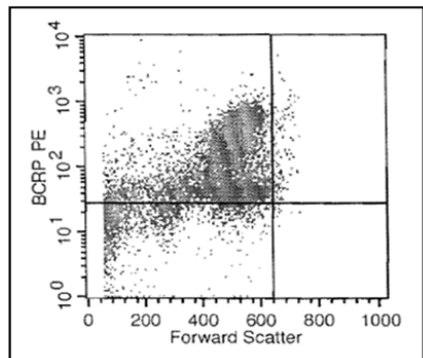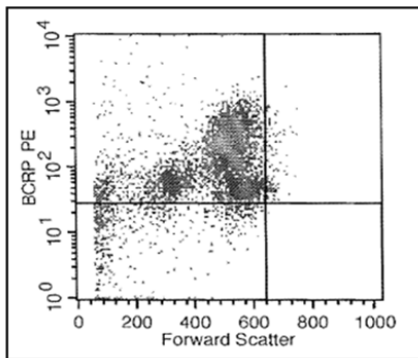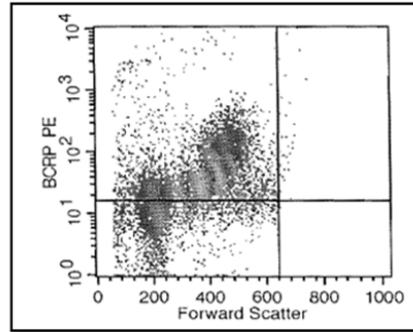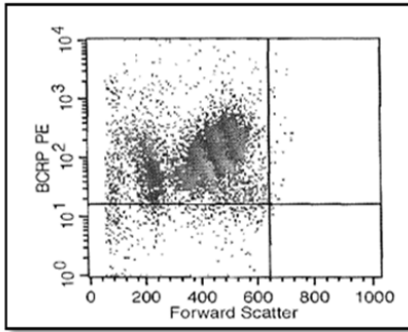i) The control group (n=5)
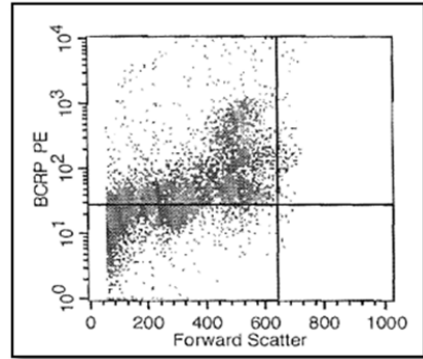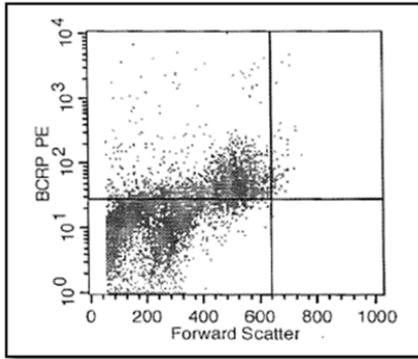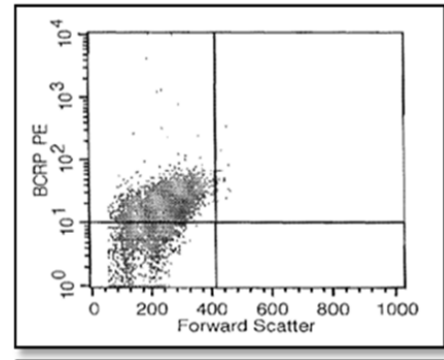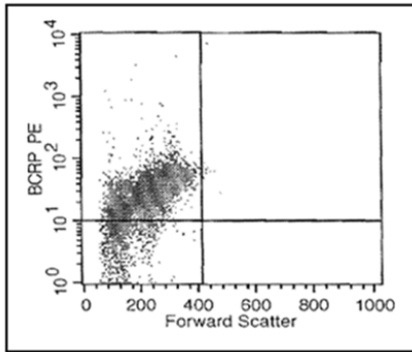
ii) The CT group (n=25)
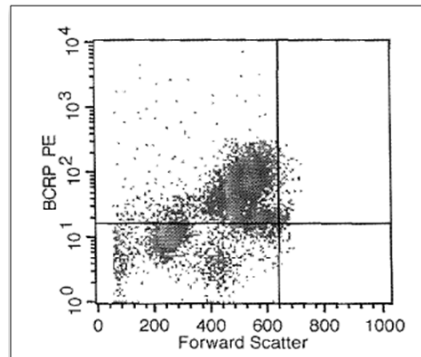*a)   At the first cycle of treatment*
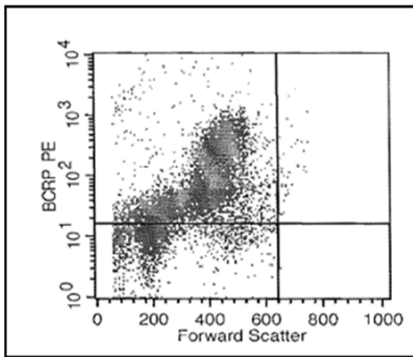

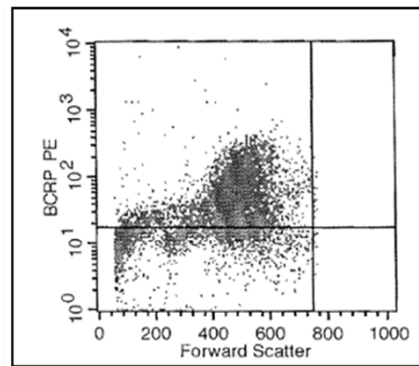*b)   At the second cycle of treatment*
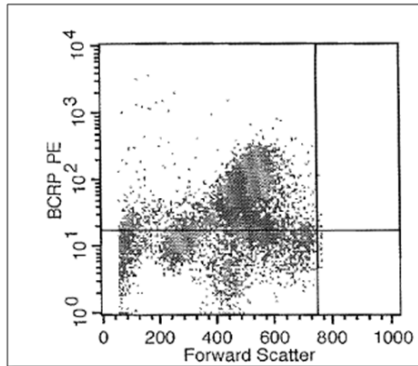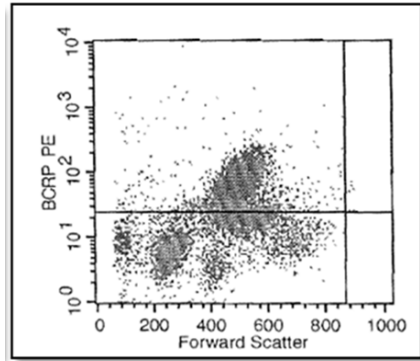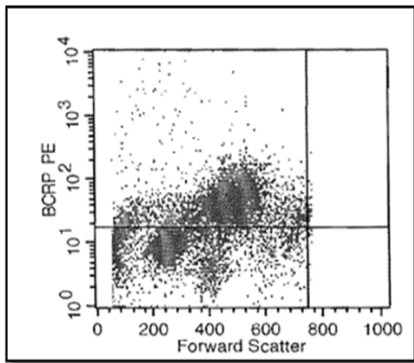
c) At the third cycle of treatment

iii) The CT plus MF group (n=25)
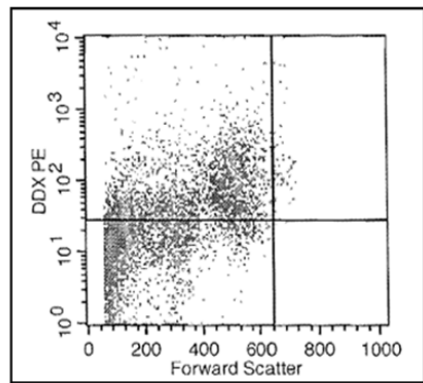*a) At the first cycle of treatment*
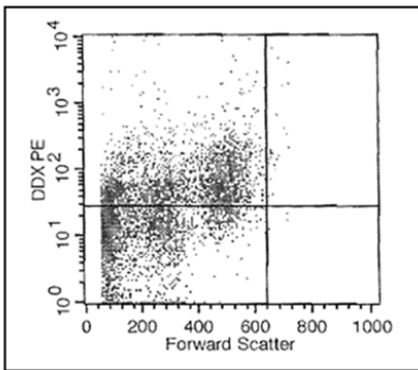

b) At the second cycle of treatment

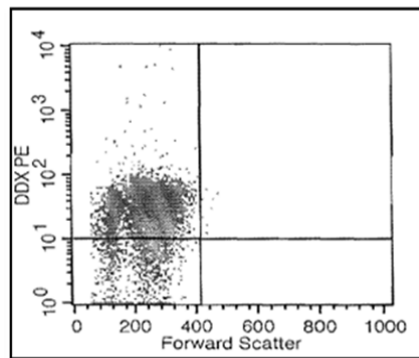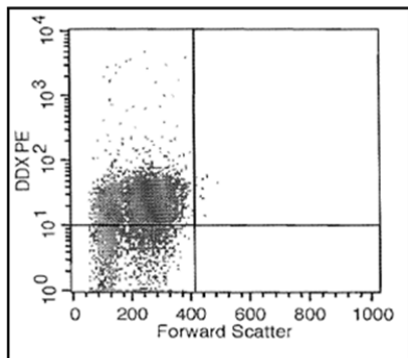
c) At the third cycle of treatment

2. Flow cytometric results of miRNA
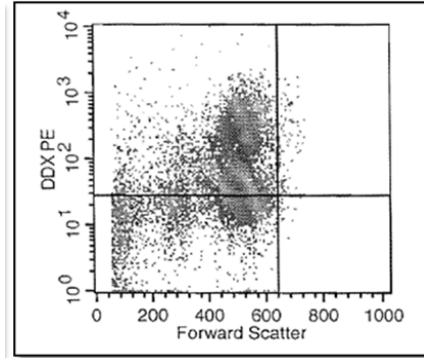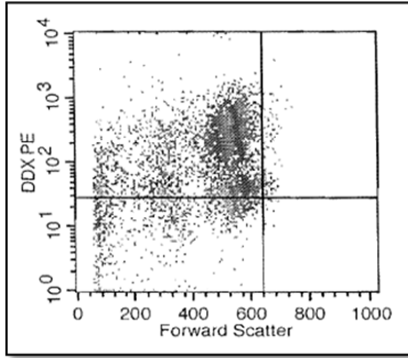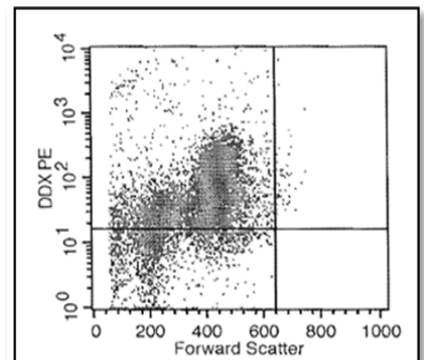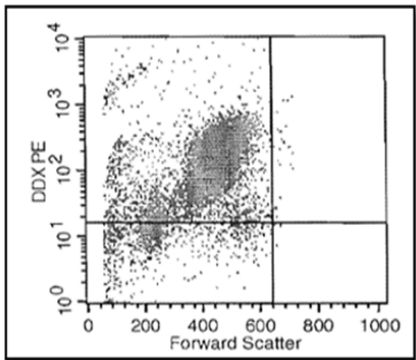
i) The control group (n=5)

ii) The CT group (n=25)

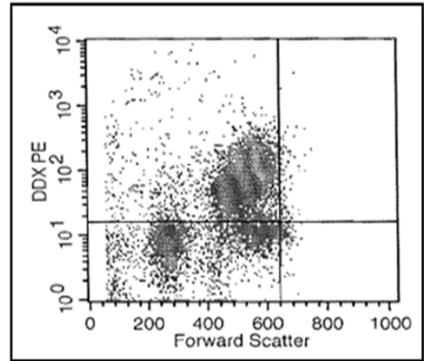*a)   At the first cycle of treatment*
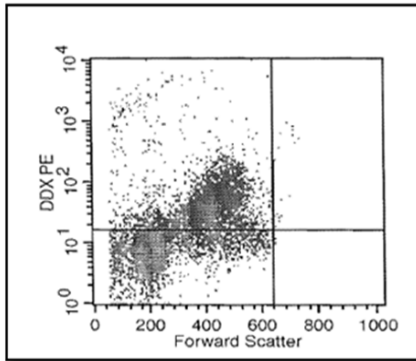

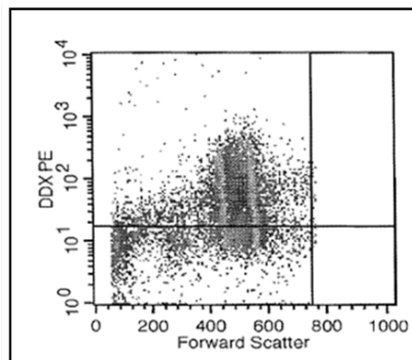*b)   At the second cycle of treatment*

c)   At the third cycle of treatment

iii)The CT plus MF group (n=25)
a)   At the first cycle of treatment

b)   At the second cycle of treatment

c)   At the third cycle of treatment
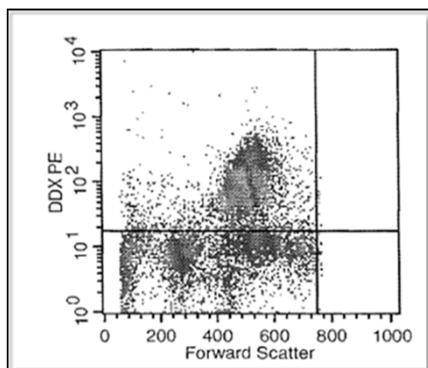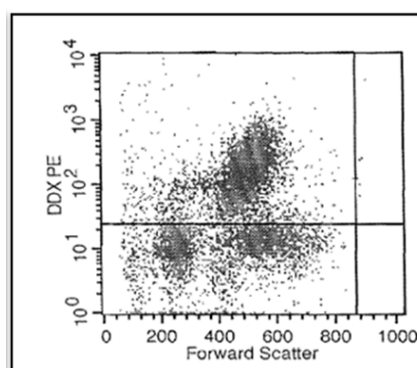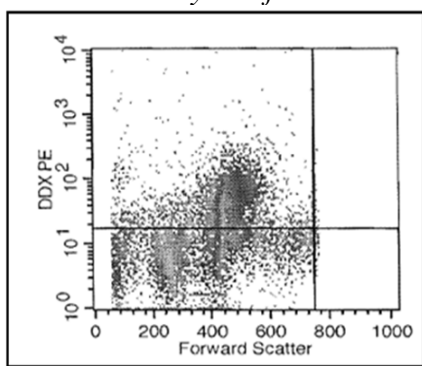

Discussion

BC is a major health problem that will affect approximately 1.3 million women worldwide. In 2013 the American cancer society, estimated that more than 296,000 women and 2,240 men were diagnosed with BC, Recently, researchers at the NCI projected that the overall BC incidence rate will stay the same through 2016. CT plays a key role in the treatment of BC; however, the widespread resistance to CT often results in the treatment failure. Drug resistance to chemotherapeutic agents accounted for treatment failure in more than 90% of patients with metastatic cancer.

Previous studies indicated that BCRP conferred an atypical MDR phenotype. A transfectant BCRP expression cell model was established and utilized to screen clinical anticancer drugs in vitro. In addition, BCRP expression was reported in 20–30% of clinical BC tissue specimens. A recent result suggested that BCRP expression may contribute to the failure of BC CT to a certain extent.

The present results showed that the control group had the lowest values for BCRP (mean: 26.29). The (CT) group had the highest values (mean: 83.03) after the third cycle and near this value post first and second doses. The MF group situated between the lowest and highest values (mean 51.87)

after third cycle. These values indicated the basic role of BCRP as MDR and reflected, in addition the success of MF in amelioration of elevated values of BCRP in the third group (CT + MF).

Present results were coincided with many literatures that revealed increased values for BCRP either in BC or in other types of cancers. Current results were coincided with this consideration stating that the occurrence of BCRP is higher in BC patients group (CT) than control group. But after the MF treatment this group demonstrated lower expression due to MF's indirect effect on the role of BCRP as a drug resistant.

With respect to miRNAs, these compounds are evolutionarily conserved, endogenous, single-stranded, noncoding RNA molecules that are reported to be involved in many biological processes, including cell proliferation, apoptosis, and tumorigenesis, through their regulation of gene expression. Most miRNAs bind to target sequences located within the 3'UTR of mRNAs by base pairing, resulting in the cleavage of target mRNAs or repression of their translation.

Regarding present results, the same values of miRNA were more or less observed in both normal and (CT + MF) groups (mean: 49.36 and 49.92 and the median: 49.0 and 49.47 respectively). The elevated miRNA values were recorded in second group (CT) at all cycles. These values indicated that the MF revealed its role in reducing miRNA, which in turn represented the improvements in this group (CT + MF) and this amelioration was dose-dependent. These results were coincided with previous reports elucidated.

Eventually miRNAs which are found intracellularly and extracellularly are functioning in mainly the regulation of gene expression, apoptosis and tumorigenesis. Moreover, the recent role of miRNAs in BC is diagnosis and treatment.

Since miRNAs were dysregulated in both normal and malignant tissues of the breast and other body fluids. Therefore their expression was varying in the same tissue with levels up and down and sometimes vice versa with respect to previous studies. So the amount of miRNA in control group was lower than that of the patients groups (group CT & MF). In addition miRNAs act as oncogenes and tumor suppressor genes and vice versa inside the cell under certain circumstances. In current results, this dysregulation of miRNA was clearly noted. Before the treatment with MF in the (CT) group miRNAs expression was found in a higher level that could be due to miRNA biogenesis pathways, in which the Cis regulation targeting of mRNA is done so in this situation the occurrence of miRNAs was highly found in the results as they may be oncogenes.

On the other hand after the addition of MF medication, MF could acts as a miRNA inhibitor. In this situation the occurrence of miRNAs was found in (CT + MF) group to be lower than the (CT) group only. This reduction in

miRNA may reflect its role as tumor suppressor gene instead of oncogene. This in turn highlighted the role of MF in targeting miRNA.

Finally the role of MF was well illustrated in targeting of miRNA to reinforce BC medication. In conclusion we can advise oncologists to use MF equivalent to CT in the recommended doses. MF led to a significant reduction in untoward effect of CT and cancer and improved the patients' outcome which may be due to its role as selectively killer for the cells that were chemoresistant.
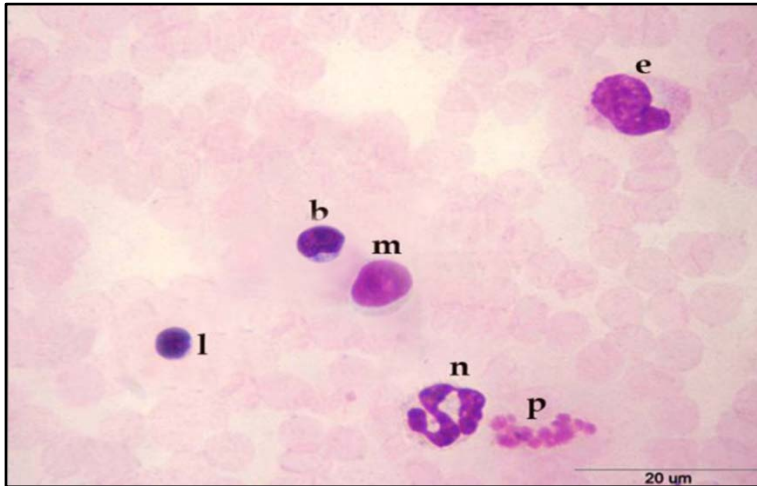
Fig. 1: Leishman's staining of control groupX1000(e:eosinophil,b:basophil,m:monocyte,l:lymphocyte,n:neutrophil, p:platelets).
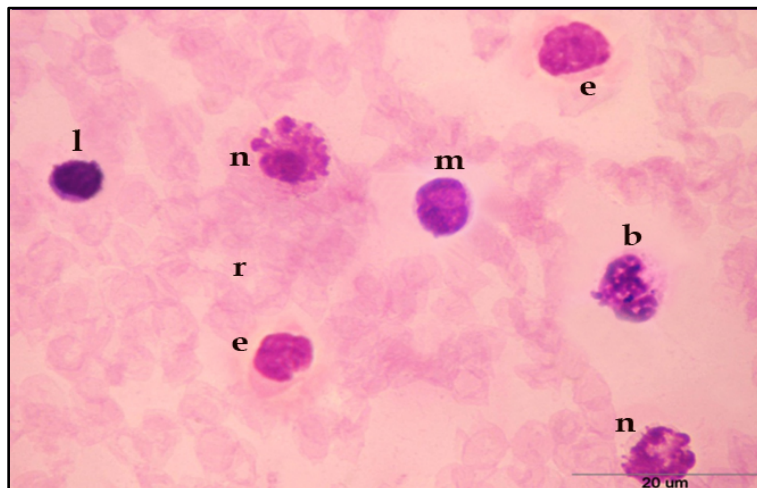
Fig. 2: Leishman's staining of CT group X1000(e:eosinophil,b:basophil,m:monocyte,l:lymphocyte,n:neutrophil,p:plat elets,r:rbcs).
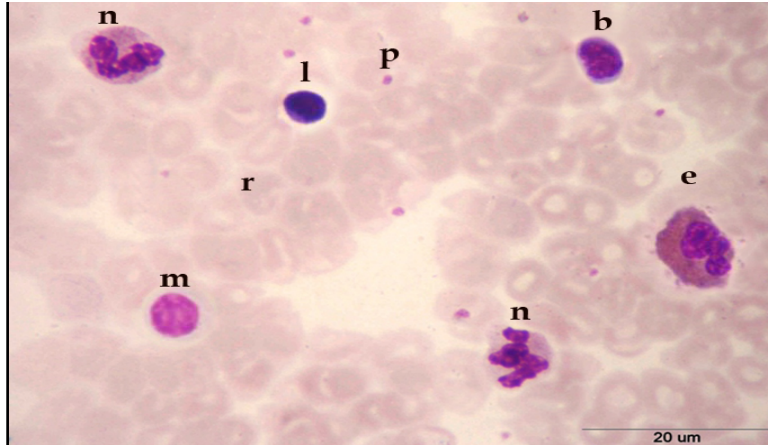
Fig. 3: Leishman's staining of MF group
X1000(e:eosinophil,b:basophil,m:monocyte,l:lymphocyte,n:neutrophil,p:plat
elets,r:rbcs).

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