Iraqi Journal of Biotechnology, 2016, Vol. 15, No. 2 , 30-41

Detection of MDR Gene (IFITM3) and P- glycoprotein Expression in Patients with Hodgkin's Lymphoma in AL-Ramadi Teaching Hospital

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Received: November 18, 2015 / **Accepted**: November 24, 2015

Abstract: The present study aimed to shed light on the follow up of Hodgkin's lymphoma patients at initial diagnosis and after treatment, to assess the response and early relapse after chemotherapy through evaluating the gene expression level of one of the major multidrug resistance genes which is the multidrug resistance 1 (MDR1),and investigate the presence of P-glycoprotein by immunohistochemistry assay. The study was conducted on fifty Hodgkin's lymphoma patients during the period extended from January 2015 to November 2015. The patients included 41(82%) males and 9 (18%) females ,with ages ranged from 12 to 81 years. The study also included ten persons (7 males and 3 females) as healthy control group . All patients were followed up by doing some laboratory test such as complete blood count before and after chemotherapy, and the tumor markers which indicate the response degree of patients to chemotherapy

DNA was extracted from paraffin embedded tissue, which were collected from AL-Ramadi teaching Hospital / Histopathology unit, in order to detect the Interferon-induced trans membrane protein IFITM3 (MDR) gene using PCR technique .The results showed in the healthy individuals, there were no mutation presence in the MDR gene in patients and control.

Results of immunohistochemistry study showed that the presence of (MDR1) P glycoprotein had played a great role in regulation of the cancer cells growth. Depending on the intensity of coloration, and percent of cancer cells stained , the results indicated the production of P-glycoprotein in 15 (30%) case as 1+, 6 (12%) case as 2+ and 10 (20%) case as 3+, while other cases 19 (38%) were negative.

Key words : Hodgkin's Lymphoma, MDR gene (IFITM3), P- glycoprotein Expression

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Introduction

Hodgkin lymphoma (HL) is a B lymphocyte malignancy (1). As hematological malignancy occurs, the lymph nodes start to grow abnormally, the presence of the mononuclear this abnormal cells are called Reed Sternberg cells which are the markers of HL. Since the lymph nodes are speared

throughout the body, cancer can initiate from anywhere inside the body (2,3). Hodgkin lymphoma is a neoplastic proliferation of lymphoid cells predominantly involving lymphoid tissues. The malignant cell is the Reed-Sternberg cell, which was first described in 1832 cited by Thomas Hodgkin, and subsequently called Hodgkin's lymphoma, which has been re-named Hodgkin lymphoma following its recognition as a clonally lymphoid disease (4). Hodgkin's lymphoma is one of the commonest lymphomas in the developed world, occurring with an occurrence of approximately 17,600 3 new cases per 100,000 person-years (Information Services Division, 2010). Hodgkin's lymphoma were diagnosed in Europe in 2013 (0.5% of total cancer cases). In Europe (2013), the highest world age-standardized occurrence rates for Hodgkin's lymphoma are found in Croatia for both men and women; the lowest rates are found in Iceland for men and Albania for women (5). In Iraq, Basrah-Southern the five leading cancers registry Hodgkin's lymphoma in 2005 - 2006.

Materials and Methods

Patients and Blood Samples

Patients and control

Fifty cases of patients with Hodgkin's lymphoma of males and females the diagnosis of biopsy by histopathology subjected to the **ABVD** chemotherapy treatment in AL- Ramadi General Teaching hospital and all patients received the defense line of chemotherapy of diagnosed cases during the period from January 2015 to March 2015 . The patients included 41 males and 9 females whose ages ranged from 12-81 years.

Tissue Biopsy

Fifty blocks of tissue biopsy from patients with Hodgkin's lymphoma were collected from AL-Ramadi General Teaching hospital .

Table (1) Primary Antibody that is used in IHC Technique

Antibody	Company	Code	Clone	Dilution
Monoclonal Mouse Anti Human P-gp	Dako Cytomation	M 7047	1D5	1 / 150

Immunohistochemistry for the Detection Surface Expression of P-gp on Paraffin Embedded Sections

Principle of the Test

Immunohistochemistry for the detection surface expression of P-gp on paraffin embedded sections in the biopsy of Hodgkin's lymphoma Patients (7).

Antigens in tissues are detected by two stages process, the binding of the primary antibody to a specific epitope, and the subsequent detection of this binding by a colorimetric reaction. The sections incubated with primary antibody. The bound primary antibody is then detected by a serial addition of biotinylated secondary antibody, horse radish peroxidase - streptavidin (HRP) conjugate and DAB substrate. The DAB (diaminobenzidine) substrate offers the greatest sensitivity of all the HRP colorimetric chromogens. The insoluble, permanent brown precipitate has a high contrast in photographs.

values of P-gp were done according to DAKO scoring system, using the following groups: **0**, negative result or membrane staining in 0 - 10 % of the cancer cells; **1+**; weak and incomplete membrane staining in 10 - 30 % of the cancer cells; **2+**; weak or moderate, complete membrane staining in 30 - 50 % of the cancer cells; **3+**; strong complete membrane staining in 50 -75 % of the cancer cells.

Molecular Study

DNA was extracted from Hodgkin's lymphoma tissue block samples using promega company , USA kit .

Detection of IFITM3 Gene

The drug resistance genes PCR kit has been designed to detect human IFITM3gene. Kit includes all necessary PCR amplification reagents with exception of Taq Polymerase, that was purchased from KAPA Taq DNA Polymerase, USA. The predicated amplicon size of human drug resistance gene IFITM3 was 116 bp . One kit of PCR (Maxim Bio. USA) was used in the present study , each one sufficient for 50 tests. The components of the kit shown in Table (2).

Table (2):PCR reaction components for amplification of IFITM3 gene

Component	Quantity (µl)	Concentration
Forward primer	1	10(pmol)
Reverse primer	1	10(pmol)
DNA template	5	10 (ng/ul)
D.W	13	-
Total volume	20	-

(Instruction manual of Kit).

The primers used for IFITM3 gene were as in table (3).

Table (3) The primers used for IFITM3 gene were used in this study

Gene	Sequence of forward primer	Sequence of reverse Primer	Product size
IFITM3 gene	F5'CCAGAATCAACGCATTGCGA3'	R5'ACAGTCAGTGATCACTGCC3'	116

PCR Protocol

Taq DNA polymerase was added as requirement of kit that provided from KAPA Taq DNA polymerase, USA. Reaction Mixture Preparation:

The reaction mixture and the test sample A that 2-steps PCR, using 94 ºC for denaturation and for annealing and extension Table (4)

Table (4) PCR Program Steps for (Human Drug Resistance Gene)

Step of PCR	Temperature programming	Time	No. of Cycle
Initial denaturation	94 ºC	1 min	1
Denaturation	94 ºC	45sec.	33
Annealing	55 - 62 ºC	45sec.	
Extension	72 ºC	150 min	
Final extension	72 ºC	5 min	1

Results and Discussion

Descriptive Data on Study Patients

Tumor markers and the Hodgkin's lymphoma tissues checked up for immunohistochemistry (**IHC**) detection of P- glycoprotein (**MDR1)** and molecular study for detection of (**MDR) IFITM3** gene by using PCR technique. This study involved 50 patients with Hodgkin's lymphoma; their mean age was 45 years with a range of (12 to 81) years. Compared with 10 persons healthy control their mean age was 30 years with a range of (15 to 50) years as show in Table (5).

Table (5): Descriptive Data of The Age Parameter of Hodgkin's Lymphoma Patients (n=50) and healthy Control (n=10)

	Max Age	Min Age	Mean Age S.D ±
Age [year] Hodgkin's Lymphoma	81	12	45 ± 3.09
Age [year] (control)	50	15	30 ± 2.58
T-test value	---	---	6.275 *

Mix.: Maximum ; Min.: Minimum. * (P<0.05).S

Distribution of Hodgkin's Lymphoma According to the Age of Patients and Scoring of P-glycoprotein

P- glycoprotein was detected in all Hodgkin's lymphoma patients, with high frequency (26.67 %) at the age group (31- 40) of patients score 3 strong , while patients with score 2 moderate represented (6.67%) , followed by age group (41–50) scoring 3 (25.00 %) of patients with score2 (12.50 %) , followed by age group >60 scoring 3 (20.00 %) of patients with score2 (10.00 %) , followed by age group(20–30) score 3 (18.18 %) of patients with score2(9.09 %) as shown in Table (6). Results showed there were a highly significant difference among the studied groups and scoring P-gp p≤0.01.

Table (6): The age frequency of Hodgkin's lymphoma patients according to the age groups and scoring p-gp

Groups	Age groups	Frequency	Scoring of intensity P-gp				
			Negative %	Weak %	Moderate %	Strong %	Chi-square %
1	20–30	11	6 (45.55) a	2 (18.18) b	1 (9.09) c	2 (18.18) B	9.268 **
2	31–40	15	4 (26.67) b	6 (40.00) a	1 (6.67) c	4 (26.67) b	9.071 **
3	41–50	8	2 (25.00) b	3 (37.50) a	1 (12.50) c	2 (25.00) b	8.941 **
4	51–60	6	2 (33.33) a	2 (33.33) a	2 (33.33) a	0 (0.00) b	10.038 **
5	>60	10	5 (50.00) a	2 (20.00) b	1 (10.00) c	2 (20.00) b	9.872 **
	Total	50	19	15	6	10	---

**** (P<0.01).**

Amplification of Multidrug Resistant Genes IFITM3 by PCR

In the Multidrug Resistant Genes, PCR method that used human Interferon-induced trans membrane protein 3 **IFITM3** gene, Master Mix contains specific primers to MDR **IFITM3** genes with ladder. This system was used in controls and patients with Hodgkin's lymphoma. A positive specific amplification was resulted in the generation of a positive specific amplification band in (116 bp) added to an internal positive control band as show in Figure (1) . While in the healthy individuals, there were no mutation presence in the MDR gene in patients and control.

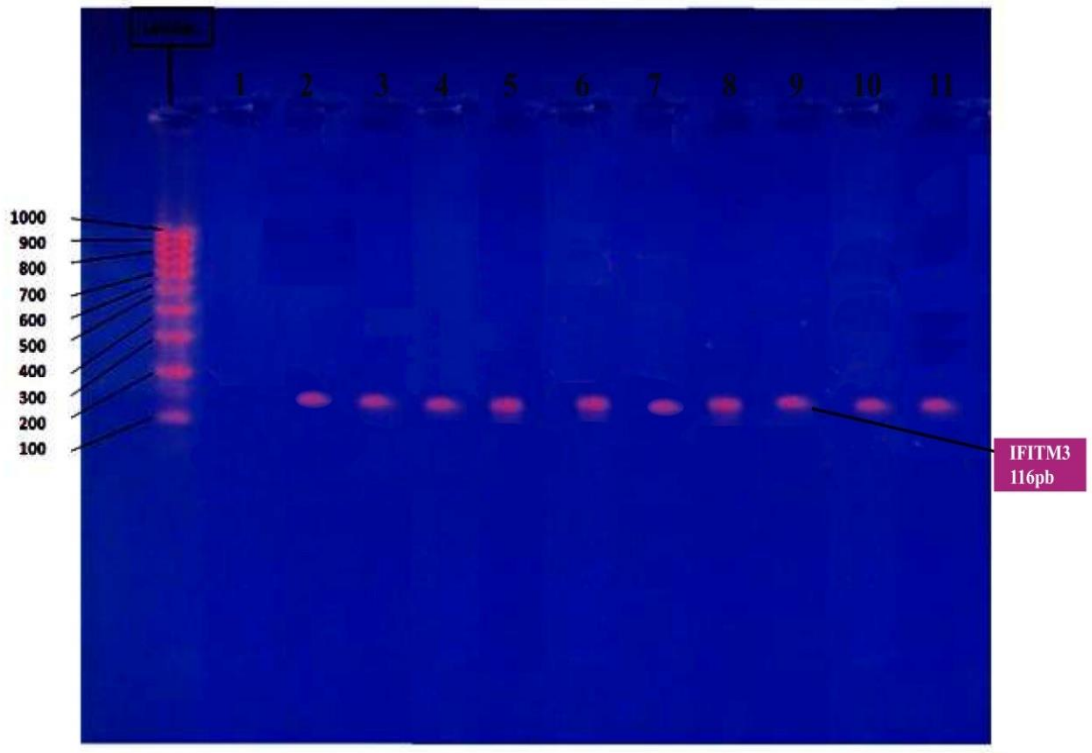

Figure (1): Gel electrophoresis of MDR(IFITM3)gene amplification by PCR products for healthy and patients with Hodgkin's lymphoma patients on 2% agarose using tissue extracted DNA, 5 volt / cm for hr. L , ladder DNA marker , 100- 1000 bp lane 1 negative control , positive healthy control and patients Hodgkin's lymphoma

Immunohistochemistry

IHC Expression of P-glycoprotein (P-gp)

The immunohistochemistry study for the P-glycoprotein over expression with Hodgkin's lymphoma revealed that score **(3+)** strong positive over expression (complete membrane staining in 50 - 75 % of the cancer cells) was present in 10 patients **(20%)** , while score **(+2)** weak or moderate, complete membrane staining in 30 - 50 % of the cancer cells was found in 6 patients (**12%**) and score **(+1)** weak and incomplete membrane staining in 10 - 30 % of the cancer cells was presence in 15 patients **(30%)** cases were not expressed or no membrane expression, score **(0) Negative** result or membrane staining in 0 - 10 % of the cancer cells, cases 19 patients **(38%)** , it scored 0. Negative and 1+ cases were considered as a normal P-glycoprotein over expression as show in (Table 7 and Figure 2).

Table (7): Frequency of immunohistochemistry staining of Hodgkin's lymphoma patients expression Multidrug Resistant P-glycoprotein

Degree of intensity	Negative (0)	weak (1+)	Moderate (2+)	Strong (3+)	Total
No. of cases	19	15	6	10	50
Percentage	38%	30%	12%	20%	100%

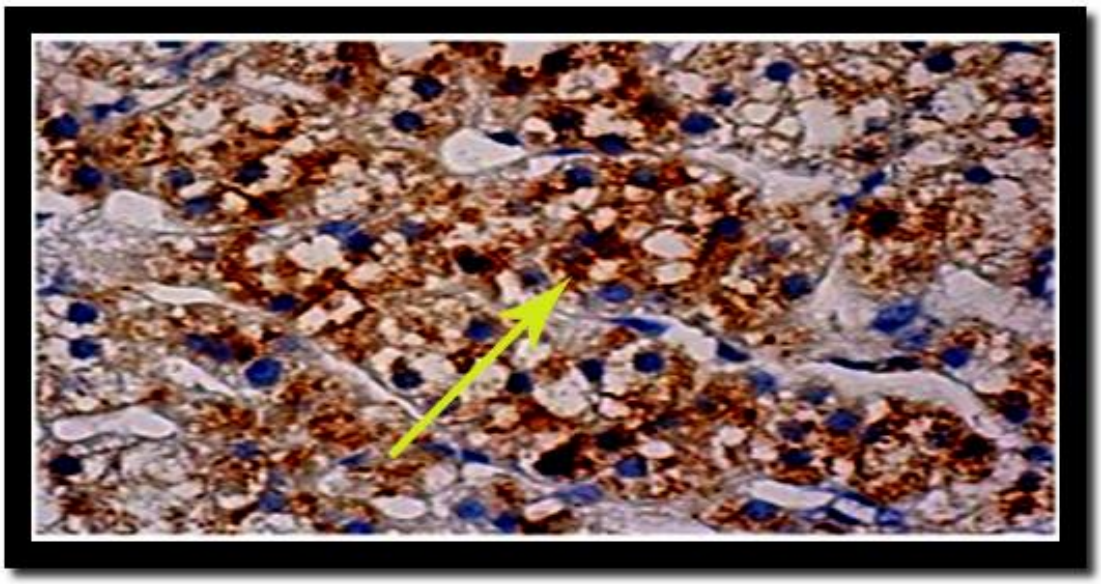

Figure (2): Immunohistochemical staining positive strong of P-gp in Hodgkin's lymphoma section by peroxidase /DAB (Brown) counter stain with hematoxyline . Total magnification is 400.

Molecular Assessment

Effects of MDR and P- glycoprotein on Clinical Response to Chemotherapy

MDR is the phenomenon in which cancer cells exposed to one anticancer drug show resistance to various anticancer drugs that are structurally and functionally different from the initial anticancer drug. The most investigated mechanisms with known clinical significance are the activation of trans membrane proteins effluxing different chemical substances from the cells (8). The human multidrug resistance P-glycoprotein (P-gp) confers drug resistance to cells because of its ability to transport a wide variety of structurally unrelated cytotoxic compounds out of the lipid bilayer of the cell (9). The impact of MDR related genes on clinical response to treatment was assessed in this study. Presence of an overt association between MDR and clinical response to treatment cannot be easily proven . Development of multidrug resistance to chemotherapy is common during disease progression . Apart from selection of the MDR phenotype by repeated chemotherapeutic treatment, it remains possible that chemotherapeutic agent might themselves directly induce P-glycoprotein over expression at the transcriptional level (10). In here , we absorbed that C3435T polymorphism is significantly associated with susceptibility to HL. C3435T polymorphism play a role in susceptibility to HL but not its response to **ABVD** chemotherapy (11). C3435T polymorphism is due to mutation at the examined locus that are related to tumor development. A variety of mechanism that may account for resistance of tumor cells to chemotherapy were described (12) . The most important one is the increase efflux of chemotherapeutic agent outside the cell by increase the expression level of the major membrane transporter p-glycoprotein (13) .The reason for multidrug resistance are , various in which over expression of the gene p-glycoprotein (MDR1) may implicate a critical role and may be the most frequent from of drug resistance in relapsed Hodgkin's lymphoma and acute leukemia (14). Apoptosis is known to play an important role in the response to genotoxic stress. Therefore, loss of apoptotic response in tumor cells is thought to be one of the mechanism involved in malignant progression and resistance to chemotherapy (15). Chemotherapy is usually not considered as a chemotherapy option and other modalities such as surgery or radiotherapy are utilized, if possible. However, in tumors showing induced type of resistance to chemotherapy, initially the cancer is chemo sensitive. Among different tumors, Hodgkin's lymphoma is thought to be moderately responsive to chemotherapy. However, to obtain a sustained curable with the combined use of surgery ,chemotherapy, radiotherapy, is not always possible since local or distant disease relapses are observed many years after the chemotherapy of the primary tumor. Then, chemo resistance becomes a real challenge to overcome especially in the unresponsive group of patients. Knowing the probability of developing drug resistance may help us to choose the type of drugs to be used in advance.

In addition, beneficial treatments in the reversal of MDR mechanisms could be added to the standard chemotherapy .In the present study, cancer samples from Hodgkin's lymphoma patients were evaluated before treatment. A single cancer sample was obtained in most of the studies. The results showed that there was a significant difference in overexpression of variant multidrug resistance genes like IFITM3 in Hodgkin's lymphoma patients compared with Verrelle reported for the first time as a direct relation between MDR1 gene overexpression and **ABVD** resistance in untreated Hodgkin's lymphoma patients. Similarly, all patients with recurrent disease had P-glycoprotein positivity, whereas high percentage of the patients with no evidence of recurrence stained negative for P-glycoprotein . The present study showed for IFITM3 genes were overexpressed in Hodgkin's lymphoma patients. All Hodgkin's lymphoma tissue samples unstained for P-glycoprotein after treatment , this may be pointed out for not choosing **ABVD** in the chemotherapy (17,18). MDR1 gene P- glycoprotein played a key role in the development of clinical resistance to treatment in other studies. Patients with induced or increased MDR1 gene overexpression were unresponsive to treatment. However, in the group of patients with ***de novo*** ABCB1expression which remained constant during treatment, clinical response was affected only in patients treated with **ABVD**. The MDRIFITM3 genes expression was found to be increased according to the type of cancers, it was obvious that inflammatory Hodgkin's lymphoma tend to be P-glycoprotein negative cancer so these groups characterized by high responsiveness to receiving adjuvant chemotherapy. Multidrug resistance (MDR) in cancer cells is a phenotype whereby cells display reduced sensitivity to anticancer drugs, based on a variety of mechanisms, including an increase in drug efflux, the reduction of drug uptake, the activation of cell growth and survival signaling, the promotion of DNA repair, and the inhibition of apoptosis signaling. Increased expression of the plasma membrane drug efflux pumps, the ATP-binding cassette (ABC) transporters, is involved in MDR. Chemotherapeutics are the most effective treatment for metastatic tumors. However, the ability of cancer cells to become simultaneously resistant to different drugs a trait known as multidrug resistance remains a significant impediment to successful chemotherapy. Three decades of multidrug resistance research have identified a myriad of ways in which cancer cells can elude chemotherapy, and it has become apparent that resistance exists against every effective drug, even our newest agents. Therefore, the ability to predict and circumvent drug resistance is likely to improve chemotherapy. The multidrug resistance to chemotherapy is an important issue for all types of cancers. Drug resistance can either be an intrinsic or an acquired form resistance. In intrinsically resistant tumors MDR related genes are constitutively expressed and these are the malignancies of tissues where these genes have a role in secretion or protection. On the other hand, acquired resistance can either be due to selection of drug resistant clones during

chemotherapy or areal induction of MDR related genes in cancer cells. In cancer types with intrinsic resistance chemotherapy is usually not considered as a treatment option and other modalities such as surgery or radiotherapy are utilized, if possible. However, in cancers showing induced type of resistance to chemotherapy, initially the tumor is chemo sensitive. Later in the course of the disease, resistance may develop disabling us to properly treat the recurrent or the metastatic disease with chemotherapy.

Detection of P-glycoprotein by Immunohistochemistry

These transporter proteins mainly located at the cell membrane work as an efflux pump driving drug molecules out of the cell, and ATP hydrolysis is coupled to the drug efflux (19). P-glycoprotein level has been shown to be useful as an indicator for predicting the clinical response chemotherapy of Hodgkin's lymphoma (20) . P-glycoprotein is useful in predicting the early relapse rate of lymphoma tissue. Relapsing Hodgkin's lymphoma patients with positive glycoprotein over expression may have also one or more of poor or failure prognostic factors (21). The level of P-glycoprotein in Hodgkin's lymphoma tissues has therefore become widely accepted as a guide for the appropriate mode of chemotherapy. There are several possible uses of P- glycoprotein status. P- glycoprotein positivity is associated with worse prognosis (increasing rate of recurrence or early relapse and mortality) . P- glycoprotein tatus might be incorporated into a clinical decision, along with other prognostic factors, regarding whether or not to give any adjuvant systemic chemotherapy. P-glycoprotein status is also predictive for several systemic therapies (22). It has generally assumed that when malignant transformation occurs, certain Hodgkin's lymphoma cells may retain all or part of the normal population of P-gp may develop into P-glycoprotein positive cancer. Most P-glycoprotein positive Hodgkin's lymphoma cells are presented in infiltrating cancer cells. In this study , p-gp was more expressed in patients who were resistance to front line treatment or those with short primary disease free interval . Patients with negative p-gp expression had a better overall response compared with those positive p-gp expression and that the density of p-gp expression was inversely related to the response rate (23) . The possible explanations could be offered that the presence of defective cytoplasmic P-glycoprotein prevent nuclear translocation of these receptors, or the absence of or defective nuclear binding site required for P-glycoprotein. One of the most significant factors which determine the level of P-glycoprotein and to smaller extent, the level of P-glycoprotein in patients with Hodgkin's lymphoma is the menopausal status of patients. Overexpression of this gene is associated with a more aggressive clinical course, a higher occurrence of clinical early relapse, and a decrease of overall survival in node positive cases. In node negative patients, P-glycoprotein overexpression is not a strong prognostic factor(22). Prognostic value of P-glycoprotein overexpression in node negative Hodgkin's lymphoma appears to become more reliable by measuring its amplification with fluorescence (24). P-glycoprotein gene overexpression, evaluated with immunohistochemistry,

it is the major predictive factor for the efficacy of brentuximab, monoclonal antibody against extracellular domain of the P-glycoprotein receptor (25). P-glycoprotein overexpression may also predict benefit from **ABVD** (26). P-glycoprotein overexpression may confer resistance to chemotherapy **ABVD**. These results were agree with AL-Ajili 2010 that P-glycoprotein scoring immunohistochemistry technique was recognizable at a high level in untreated breast cancers, increased expression of P-glycoprotein may be involved in the natural resistance of tumors to anticancer agents.

Conclusions

Based on the finding of the present study, it is concluded that:

The demographic features of our patients with Hodgkin's lymphoma consistent with worldwide studies. No mutation detected in (IFITM3) gene in both patients and control. The detection of expression p-glycoprotein there were highly significant differences in the scoring of and P- glycoprotein expression among show that scoring negative (0) 38% , 1+ weak 30% **,** 2+ moderate 12% and strong +3 20%.

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