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RESEARCH ARTICLE

Do Gamma Rays of Cancer Radiotherapy Effect on the Sequence of Collagenase Gene?

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Key words:-
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Abstract

Background: expression of certain HLA alleles, considered susceptible factor to acute leukemia (AML, ALL), there were clear associations between resistance or susceptibility to cancer and (HLA) profile**,** this work **aims to** investigate role HLA-DRB1 and anti HLA–G in development of AML. **Patients and methods:** (40) patients with acute leukemia aged from 4 to 60 years (23 male and 17 female), and 20 healthy subjects as a control, all were tested for anti-HLA-G by four-color flow cytometer using FACS Canto**(BD Biosciences: USA**), HLA-DRB1by Sequence Specific Oligonucleotide PCR (SSO-PCR), DNA purification by (Spin Protocol), Qiagen, Hilden, Germany, QIA amp DNA Mini, thermal cyclers BIO-RAD PTC-100 (USA), hybridization with DNA probes, alleles detection; INNO-LiPA HLA typing, Fuji Rebio Group, Belgium, with the INNO-LiPA HLA-DRB1 Plus kit, Cat. No. #58355. **Statistical analysis:** SPSS 19 edition was used, gene frequencies of HLA-DRB 1alleles were calculated by direct count. **Results:** there is an allelic association between HLA-DRB1 and leukemia, patients and control showed significant difference between DRB1*13 ($p = 0.006$ and $p = 0.016$ respectively) (protective nature), but there were moderate difference among DRB1*06 ($p = 0.098$), DRB1*14 ($p = 0.098$), (also, protective nature) and *15 ($p = 0.098$) (Risk factor). However, we did not observe any significant difference for the others HLA-DRB1. **Conclusion:** HLA-DRB1*13, and HLA-DRB1*15 alleles were protective while HLA-DRB*06 and DRB1*14 were predisposing factor for AML**.**

Introduction:-

Leukemia is uncontrolled proliferation of hemopoietic cells **(1)**. Acute leukemia is divided into a number of different subtypes based upon clinical, morphological, immunophenotyping, cytochemical, cytogenetic and molecular biology or by combinations of these characteristics **(2).**Although a study of HLA understanding of that relationship has been slow to emerge **(3).** Studies show that HLA genes are involved in various mechanisms of pathogenesis and immunoediting of hematological diseases **(4).** Leukemia was the first disease in which involvement of the major (MHC) was reported **(5).**Studies on the role of the HLA in leukemia susceptibility represented a lot of data with many questions were raised but few confirmatory answers were available **(6)**.Although, not all studies of leukemia and HLA demonstrate associations. Further studies are required to support these conclusions **(7).** Studies of the

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correlation between HLA polymorphisms and susceptibility or resistance to disease started soon after serological methods for HLA class I had been standardized (8).

There are clear associations between resistance or susceptibility to cancer and (HLA) profile of an individual (9).The association between HLA and various hematological malignancies has been studied extensively (10).

The rate of expression of certain HLA alleles is considered among the factors of susceptibility to develop leukemia. Many studies have been performed in humans investigating the role of HLA antigens in pathogenesis of hematological malignancies. Although, the association of HLA with the molecular features of leukemic cells is still unclear (11). HLA-G contributes to tumor escape host responses, and its potential clinical relevance in various malignancies was discussed (12). The part devoted to HLA-G in tumor escape remains to be more precisely determined in these acute pathologies (13).

Patients and methods:-

Design and population:-

This prospective study has been carried out in Hematology Unit in Clinical and Chemical Pathology department of Sohag University Hospital in the period from January, 2012 to August, 2015.Study was conducted on forty (40) patients; 23 males (57.5%) and 17 females (42.5%), suffering from acute leukemia of both lymphoid and myeloid lineage, they aged from 4 to 60 years old. The control group consisted of 20 unrelated healthy volunteers. The control panel reasonably corresponds to the same age, sex distribution, living in the same geographical area and with the same ethnic origin of the patients' panel.

Ethics:-

This study was conducted in accordance with the ethical standards of the Helsinki Declaration of 1964, as revised in 2000 and was approved by the Institutional Review Board of the Faculty of Medicine, Sohag University. Informed consent was obtained from all the study participants.

Sampling: Blood samples were collected from all patients and healthy subjects. 2 ml of EDTA anticoagulated blood were collected. Diagnosis of leukemia was based on complete blood count, blood film morphology, B.M examination cytochemistry and immunophenotyping.

Flowcytometry for anti HLA-G: Four-color flow cytometer; FACS Canto (BD Biosciences, USA was used. Purified mouse anti-human HLA-G denatured: FITC goat anti-mouse IgG/IgM0.5 mg polyclonal Cat. No. # 555988. Purified mouse IgG1, κ isotypic control 0.1 mg, MOPC-21Cat. No. # 555746. FACS lysing solution 10X ; BD Cat. No. # 349202. Falcon disposable 12 x 75-mm capped polystyrene test tubes B.D Cat. No. #2058.B.D. Monoclonal antibodies to human cell surface antigens (anti-CD33, anti-CD13 and anti-CD19 stained with Fluorescein isothiocyanate (FITC) and anti-CD45 antibodies stained with phycoerythrin (PE)).

Flowcytometry for anti HLA-G was carried out in Hematology Unit in Clinical and Chemical Pathology Department of Sohag University Hospital.

HLA Typing:-

All patients and controls were subjected to molecular HLA-DRB1 typing. 2ml EDTA-anticoagulated blood samples were collected from patients and controls. Samples stored by freezing to – 80C^{o} then thawed in 37 C^{o} in the incubator in the day of the run. Samples were processed for detection of HLA-DRB1 alleles by Sequence Specific oligonucleotide PCR (SSO-PCR) in following steps : DNA extraction using the spin column technique (QIAamp DNA Blood Mini kits; Qiagen, Hilden, Germany) was performed according to the manufacturer's guidelines; polymerase chain reaction (PCR) amplification of (exon 2) of the HLA-DRB1 alleles (INNO-LiPA HLA-DRB1 Amplification Plus kit; Fuji Rebio Europe, Ghent, Belgium) were performed and the amplicons were chemically denatured to form single-stranded DNA. Reverse dot-blotting was performed on a nylon membrane (INNO-LiPA HLA-DRB1 Plus strips, Fuji Rebio Europe), which contains an array of immobilized, sequence-specific oligonucleotide (SSO) probes. The biotin-labeled amplicons were then bound (hybridized) to these SSO probes, which contain a complementary target sequence and, thus, were captured onto the membrane strip; 3) visualization of the results was achieved by incubating with an enzyme conjugate (streptavidin and alkaline phosphatase), which binds to the biotin of the PCR product, followed by the addition of a substrate. The bands with the captured PCR product turned blue. Interpretation was achieved by using standard tables with the help of specialized software:

LiRAS™ FOR LiPA HLA V6.0X.

HLA typing was carried out in Immunology Unit in Clinical and Chemical Pathology Department of Mansoura University Hospital.

Statistical Analysis:-
Statistical analyses were carried out using the SPSS® statistical package. Released 2010. IBMSPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp., Microsoft Office Excel 2013 for Windows and online analytical computer assisted sites.
Gene frequencies of the HLA-DRB1 alleles of both study groups were calculated by direct count. For comparison between the groups, we used $\chi 2$ analysis by using 2×2 contingency tables and Fisher's exact test when appropriate; *P* values less than 0.05 were considered statistically significant. For estimating risks, we employed odds ratio (OR) with a 95% confidence interval (95% CI).

Results:-
The polymorphism of HLA-DRB1, genes was investigated in twenty normal individuals and forty leukemic patients. The clinical data of the examined subjects were presented in table (1)

Comparison of the results between the normal population and acute leukemia patients revealed that there is no difference between anti HLA-G antibodies in both patients and control groups as in table (2) figure (1,2 and), there is an allelic association between certain HLA-DRB1, and leukemia disease. Our results showed that in leukemic patients and in normal individuals, the difference between DRB1*13 was significant (p = 0.006 and p = 0.016 respectively) (protective nature), but there were moderate difference among DRB1*06 (p = 0.098), DRB1*14 (p = 0.098), (also, protective nature) and DRB1 *15 (p = 0.098) (Risk factor). However, we did not observe any significant difference for the others HLA-DRB1.

As regard gender the HLA-DRB1*10 allele was not detected in any of the male patients (P = 0.091). With no significance difference between males and females in other HLA-DRB1, illustrated in figure (3).According to the immunophenotyping of leukemia, the HLA-DRB1*01 allele was not detected in any of the AML patients in relation to ALL patients (P = 0.109). With no significance difference between AML and ALL in other HLA-DRB1.The HLA-DRB1*14 allele was not detected in any of the controls in relation to ALL patients with moderate significance (P = 0.057) (relatively risk factor). The HLA-DRB1*13 show high frequency in controls in relative to ALL patient with moderate significance (P = 0.054) (Relatively Protective). With no significance difference between ALL and controls in other HLA-DRB1 as demonstrated in table -3 and 4.

HLA-DRB1*06allele was not detected in any of the controls in relation to AML patients that significant difference (P = 0.044) (risk factor). The HLA-DRB1*13 show high frequency in controls in relative to AML patient (P = 0.006) (Protective nature). With no significance difference between AML and controls in other HLA-DRB1**.**

Discussion:-
The first study on HLA in human leukemia demonstrated an increased frequency of HLA-A2 in ALL in 1967 .From that date many previous studies have demonstrated some significant differences in HLA allele frequencies in leukemic patients and normal subjects which had reported by Diase et al (2015) and others (14, 15,16). In our study, significant positive association with the disease, in patients compared to controls, was found for two alleles: HLA-DRB1*013.Also there were moderate difference among DRB1*06 DRB1*14, and *15. It is suggested that HLA-DRB1*13, and HLA-DRB1*15 alleles may play a protective genetic factor against leukemia while the HLA-DRB1*06 and –DRB1*14 allele could be a presumptive predisposing factor. Regarding anti-HLA-G antibodies analysis, no significant association was found between patients and control groups. Also, our study shows that HLA-DRB1*10 allele was not detected in any of the male patients with moderate significance with no significance difference between males and females in other HLA-DRB1 or anti-HLA-G antibodies. Our results are consistent with Du et al (2013), that demonstrated that the allele frequencies of DRB1*15 obviously decreased in patients with leukemia so, it can be considered as genetic indicators for resistance of leukemia. But they demonstrated that the DRB1*07 have a protective role too (17). Diase et al (2015) had reported that HLA-DRB1*13, which showed a decrease in patients, should be protective against ALL (14). The findings that were demonstrated also by Dorak et al (2013),who reported that the frequency of the HLA-DRB1*13 allele was lower in male patients of leukemia (15).

Also Bosen et al (2013); reported that the frequency of the HLA-DRB1*13 allele was lower the female ALL patients compared to controls (7). On the other hand, Chaing et al (2012) had found that there is significant positive association with the disease, in patients compared to controls, was found for three alleles: HLA-DRB1*13 and HLA-DRB*01 (10). Also there were moderate difference among DRB1*04, *03 and *15.It is suggested that HLA-DRB1*13 (on contrast to our results), *03 and *04 alleles may play a presumptive predisposing factor while the HLA-DRB*01 and *15 (on contrast to our results) alleles could be a protective genetic factor against leukemia (16). A significant increase in the frequency of the HLA-DRB1*04 allele in the overall and ALL patients, was reported in many studies although there is no significant difference between patient and controls (17).We found that the HLA-DRB1*14 allele was not detected in any of the controls in relation to ALL patients with moderate significance (relatively risk factor). There is no significance difference between ALL and controls in other HLA-DRB1 or anti-HLA-G antibodies. Our results are consistent with Ehernberg et al (2014); who found, significant decrease in HLA-DRB1*04 (in contrast to be previously reported by Bosen et al (2013), and an increase in allelic distribution of HLA-DRB1*03 in ALL patients in relative to controls which show no significant difference in our study(7,18).

In our study, The HLA-DRB1*06 allele was not detected in any of the controls in relation to AML patients that significant difference) (risk factor). The HLA-DRB1*13 show high frequency in controls in relative to AML patient (protective nature). There is no significance difference between AML and controls in other HLA-DRB1 or anti-HLA-G antibodies.

Our findings are inconsistent with Elansary et al (2015), who reported that high significant increase allelic distribution in HLA-DRB1*11 and moderate decrease in HLADRB1*07 in AML patients in relative to controls which show no significant difference in our study (19).

Bosen et al (2013), observed that the frequencies of the HLADRB1*15 alleles in patients with AML were significantly higher in AML patients compared with healthy controls, Du et a (2013), observed significant increase in allelic distribution of HLA-DRB1*07 in AML patients and suggests that it is susceptible to the disease in contrast to Ehernberg al (2014) observations. These results not consistent with Chaing et al (2012) that demonstrated that allelic distribution of the HLA-DRB1*13 is significantly high among AML and overall acute leukemia patients group in relation to controls (7, 10, 17 and18). Few studies had been performed on HLA-G expression in different types of leukemia patients, however, data are limited and conclusions remain controversial and discordant (19).Regarding anti-HLA-G antibodies analysis, no significant association was found between patients and control groups. Also our results are inconsistent with the results of other authors who have not found membrane bound or cytoplasmic HLA-G expression (7).A group of previous studies addressed that no cell surface HLA-G was expressed in various types of hematopoietic diseases, such as AML, ALL, CLL and CML. In contrast to these previous studies, B-CLL samples express cell surface HLA-G antigen in a variable proportion of leukemic tumor cells. Methodology difference address this controversy (20).The discrepancy between the results of different studies, including our results, may be attributed to the differences in population race and geographical distribution. However, further larger studies are required to support our findings.

Conclusion:-

Some HLA alleles are associated with an increased susceptibility while others are protective. Although, some of the results from the present study support these earlier findings. The discrepancy between the results of different studies, including our results, may be attributed to the differences in population race and geographical distribution, environmental, occupational factors.

Conflicts of interest:-The authors had no conflicts of interest to declare in relation to this article.

Table (1):- Clinical data of the studied groups

Variables	*Control*	*Patients*
Age (years) *Mean± SD*	*32.35 ± 19.07*	*33.08 ± 21.79*
Male/Female	*11/9*	*23/17*
Diagnosis and Frequency	*100 % healthy normal*	*AML (22) 55%* *ALL (18) 45%*
Total	*20*	*40*

Table (2):- Anti-HLA G positivity between cases and controls

Item	*Anti HLA-G*		*Total*
	Negative	*Positive*	
Patients number	*17*	*23*	*40*
% within group	*42.5%*	*57.5%*	*100.0%*
Control number	*6*	*14*	*20*
% within group	*30.0%*	*70.0%*	*100.0%*
Total number	*23*	*37*	*60*
% within group	*38.3%*	*61.7%*	*100.0%*

Table (3):- HLA-DRB1 alleles in acute leukemia patients compared with healthy control.

Allele	*P value*	*Odd's ratio*	*Confidence interval of odd's*	
			Minimum	*Maximum*
1	*0.534 (NS)*	*2.662*	*0.122*	*58.122*
2	-	-	-	-
3	*0.686 (NS)*	*1.286*	*0.381*	*4.343*
4	*0.680 (NS)*	*0.778*	*0.236*	*2.568*
5	*0.383 (NS)*	*3.827*	*0.188*	*77.769*
6	*0.219 (NS)*	*6.352*	*0334*	*120.845*
7	*0.638 (NS)*	*1.417*	*0.332*	*6.048*
8	*0.716 (NS)*	*1.541*	*0.150*	*15.83*
9	-	-	-	-
10	*0.534 (NS)*	*2.662*	*0.122*	*58.122*
11	*1.000 (NS)*	*1.000*	*0.290*	*3.454*
12	*0.789 (NS)*	*1.557*	*0.061*	*39.948*
13	*0.008 (S)*	*0.206*	*0.065*	*0.660*
14	*0.219 (NS)*	*6.352*	*0334*	*120.845*
15	*0.108 (NS)*	*0.333*	*0.087*	*1.272*
16	*0.789 (NS)*	*1.557*	*0.061*	*39.948*

S: Significant NS: Non significant

Table (4): Gene frequency of HLA-DRB1 alleles corresponding to ALL and AML patients

HLA-DRB1 alleles	*Allele positivity among ALL (no. = 18)*	*Allele positivity among AML (no. = 22)*
1	*2(11.11%)*	*0*
2	*0*	*0*
3	*5(27.78%)*	*7(31.81%)*
4	*4(22.22%)*	*6(27.27%)*
5	*1(5.56%)*	*2(9.09)*
6	*1(5.56%)*	*4(18.18)*
7	*5(27.78%)*	*3(13.64)*
8	*1(5.56%)*	*2(9.09)*
9	*0*	*0*
10	*0*	*2(9.09)*
11	*4(22.22%)*	*6(27.27%)*
12	*1(5.56%)*	*0*
13	*7(38.89%)*	*6(27.27%)*
14	*3(16.67%)*	*2(9.09%)*
15	*2(11.11%)*	*3(13.64%)*
16	*0*	*1(4.55%)*

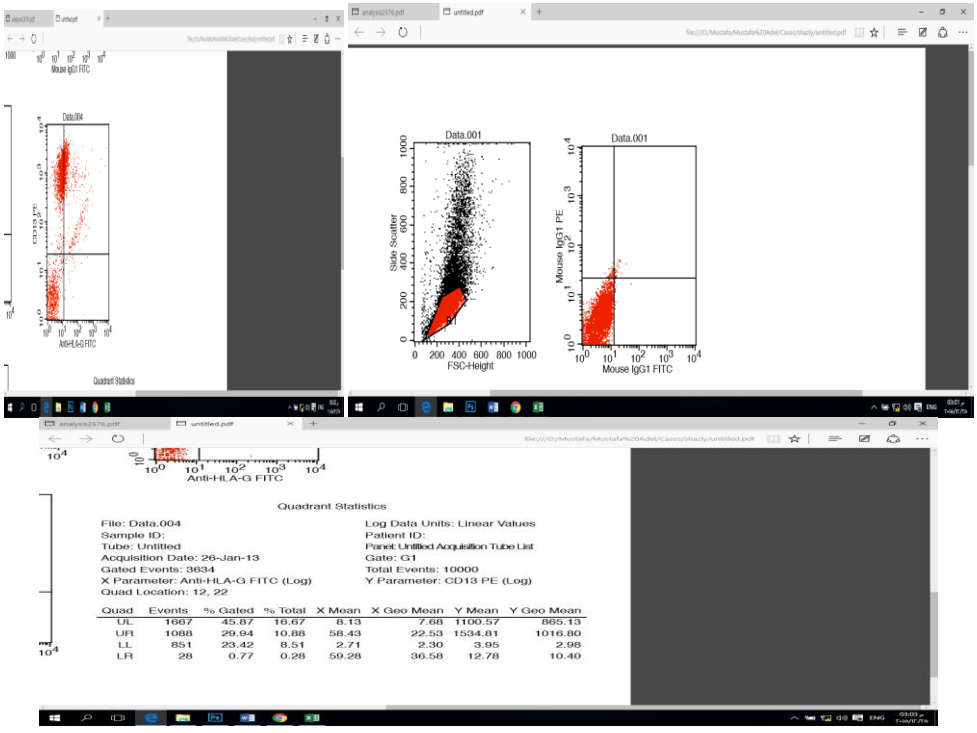

Fig (1):- Case No. 27: AML Patient with HLA-G Positive

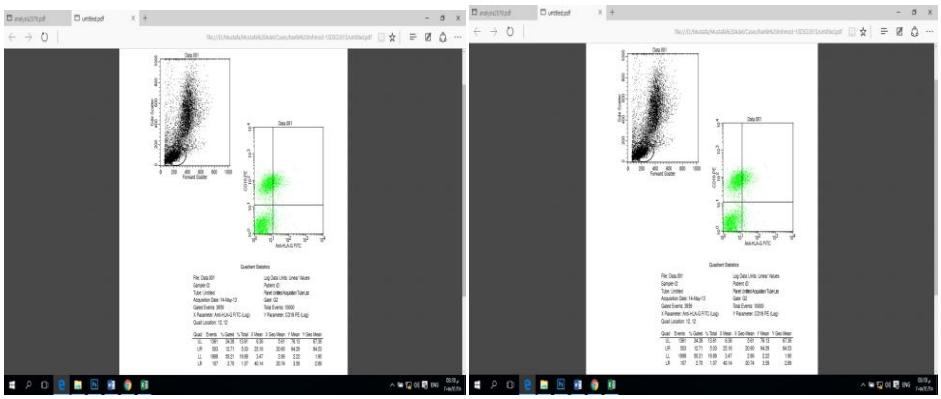

Fig (2):- Case No. 28: ALL Patient with HLA-G Positive.

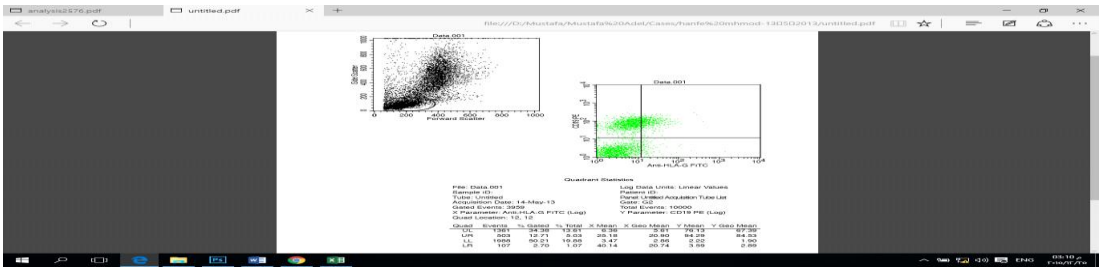

Fig (3): Odd ratio of HLA-DRB1 alleles corresponding to males and females frequency

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