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**Screening the potential role of killer immunoglobulin receptors genes among individuals vaccinated against hepatitis b virus in Lebanon**

Melhem NM *et al*. KIR and protection against HBV

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**Abstract**

**AIM:** To explore the role of killer immunoglobulin receptors (*KIR*) genes in responsiveness or non-responsiveness to vaccination against hepatitis B virus.

**METHODS:** We recruited 101 voluntary participants between March 2010 and December 2011. Sera samples from vaccinated and non-vaccinated participants were tested for the presence of anti-HBs antibodies as a measure of protection against hepatitis B, hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) as indicators of infection by enzyme linked immunosorbent assay. *KIR* genes frequencies were determined by polymerase chain reaction (PCR). Linkage disequilibrium (LD) analysis between pairs of gene loci was performed.

**RESULTS:** Sera samples from 99 participants were tested for the levels of anti-HBs as an indicator of protection (≥ 10 mIU/ml) following vaccination as defined by the WHO international reference standard. Among the vaccinated participants, 47% (35/74) had anti-HBs titers above 100 mIU/ml, 22% (16/74) with anti-HBs ranging between 10-100 mIU/ml, and 20% (15/74) with values of less than10 mIU/ml. We report the lack of significant association between the number of vaccine dosages and the titer of antibodies among our vaccinated participants The inhibitory KIR2DL1, KIR2DL4, KIR3DL1, KIR3DL2, and KIR3DL were detected in more than 95% whereas KIR2DL2, KIR2DL3, KIR2DL5 (KR2DL5A and KIR2DL5B) were expressed in 56%, 84%, and 42 % (25 % and 29%) of participants, respectively. The observed frequency (OF) of the activating KIR genes ranged between 35% and 55% except for KIR2DS4, detected in 95% of the study participants (40.6% 2DS4\*001/002; 82.2% 2DS4\*003/007). KIR2DP1 pseudogene was detected in 99% of our participants whereas KIR3DP\*001/02/04 and KIR3DP1\*003 had frequencies of 17% and 100%, respectively. No association between the frequency of *KIR* genes and anti-HBs antibodies was detected. When we compared the frequency of *KIR* genes between vaccinated individuals with protected antibodies titers and those who lost their protective antibody levels, we did not detect a significant difference. KIR2DL5B is significantly different among different groups of vaccinated participants (group I > 100 mIU/ml, group II 10-100 mIU/ml, group III < 10 mIU/ml and group IV with undetectable levels of protective antibodies). Our data show that almost all *KIR* genes with significant positive LD was between B haplotypes whereas those with significant negative LD were between A and B haplotypes.

**CONCLUSION:** To our knowledge this is the first study screening for the possible role of *KIR* genes among individuals vaccinated against HBV. Our results can be used to design larger studies to better understand the role of *KIR* genes in protection against or susceptibility to HBV post vaccination.

**Key words:** Natural killer cells; Hepatitis B virus; Killer immunoglobulin receptors; Hepatitis B vaccine; Lebanon

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**Core tip:** Currently, there are no data supporting the use of booster doses of hepatitis B vaccine among immuno-competent individuals responding to a complete primary vaccination regimen. Importantly, 5%-10% of healthy adults does not generate protective levels of antibodies and are hence considered non-responders. This study aims to explore the role of killer immunoglobulin receptors genes in responsiveness or non-responsiveness to vaccination against hepatitis B virus.

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**INTRODUCTION**

Infection with hepatitis B virus (HBV) results in a spectrum of clinical outcome ranging from acute hepatitis to end-stage liver disease and hepatocellular carcinoma[1] with an estimated lifetime risk of 25%-40%[2]. Booster studies suggest that memory begins to decline 15 years following vaccination among adolescents vaccinated at infancy[3-6]. Other studies suggest the persistence of immune memory for 20 years or longer[7-9]. Currently, there are no data supporting the use of booster doses of hepatitis B vaccine among immuno-competent individuals responding to a complete primary vaccination regimen (3 doses). Importantly, 5%-10% of healthy adults does not generate protective levels of antibodies and are hence considered non-responders[10]. Consequently, long-term protection is still debatable[11] and not linked to genetic factors.

Natural killer (NK) cells are known to induce antiviral and antitumor immunity via production of pro-inflammatory cytokines and lysis of infected or transformed cells[12]. Killer immunoglobulin receptors (*KIR*) genes encode receptors expressed on NK cells. Based on the gene content, two groups of KIR haplotypes are known in humans: A and B. Haplotype A encodes inhibitory receptors and consists of nine genes (3DL3, 2DL3, 2DP1, 2DL1, 3DP1, 2DL4, 3DL1, one activating (2DS4), 3DL2, and 2DL5) whereas haplotype B carries a variety of gene combinations and encodes more activating receptors as compared to haplotype A (3DL3, 2DS2, 2DL2, 2DL5B (inhibitory) 2DS3, 2DP1, 2DL1, 3DP1, 2DL4, 3DS1, 2DL5A (inhibitory) , 2DS5, 2DS1, and 3DL2)[13].

KIR3DS1- and KIR3DL1-expressing NK cells were reported to expand in acute and chronic human immunodeficiency virus (HIV)-1 infection, respectively[14]. Similarly, reports suggest that KIR2DL2 and or KIR2DL3 along with their ligand HLA-C1 are associated with severe influenza infection; in addition, the frequency of KIR3DS1, KIR2DS5 and KIR2DL5 was also related to the severity of the disease[15]. KIR2DS2 and KIR2DS3 were found to be associated with susceptibility to chronic hepatitis B infection whereas KIR2DS1, KIR3DS1 and KIR2DL5 may act as protective genes leading to viral clearance among the Chinese Han population[16]. A difference between the frequency of different KIR haplotypes among chronically infected individuals and those spontaneously recovering from HBV infection was also demonstrated in this population[17]. Recently, the rate of KIR2DL3 and 3DS1 were reported to be higher in healthy Turkish individuals as compared to patients with chronic HBV and those with spontaneous remission[18]; authors suggested the possible role of these genes in protection against HBV infection. In addition, genetic factors have been reported to play a role in the regulation of post-vaccine immune responses[19]. This was observed with antibody responses to a number of vaccine antigens including hepatitis B.

It is clear that the interaction between KIRs and their corresponding HLA ligands is implicated in differential responses to HIV, hepatitis C virus (HCV) and HBV as well as other disease conditions[20-23]. Immune responses, like many biological responses, are characterized by a wide range of variation between individuals. This has been described following natural infection or in response to vaccination[24]. HLA, cytokines, toll-like receptors and related gene variants were associated with a variety of immune responses following vaccinations. Recently, single-nucleotide polymorphism associations were described to be involved in innate and adaptive immune response regulation following measles and rubella vaccines[25,26]. Similarly, a difference in gene expression was also reported between high and low responders to smallpox vaccine[27]. The fact that antibody responses to hepatitis B vaccine is non-protective in up to 10% of individuals[10] and that a genetic basis to non-responsiveness is reported[19,24], prompted us to explore the role of KIR genes in response to hepatitis B vaccine in a cohort of healthy vaccinated Lebanese adults.

**MATERIALS AND METHODS**

***Study participants and samples***

Human subject approval was obtained for this study from the institutional review board of the American University of Beirut and all the methods were carried out in accordance with the approved ethical guidelines. A written informed consent was signed by the study subjects before participation in the study. A data collection form was administered to the study participants (≥ 18 years old) to collect demographic information and data related to exposure and risk behavior information.101 subjects were recruited during March 2010-December 2011. Subjects were excluded if they have had a prior or current history of HCV, HIV-1, renal disease or cancer. Children or adolescents of HBV carrier mothers and vaccinated at infancy were not included in the study.Blood was drawn from the study participants and peripheral blood mononuclear cells (PBMCs)[28] and sera were collected and stored in liquid nitrogen and at -80 oC, respectively. DNA was extracted from whole blood of the study participants using the QIAamp DNA Blood Midikit (Qiagen, Germany), as per manufacturer’s instructions. The integrity of the purified DNA was checked by gel electrophoresis and stored at -20 oC.

***Enzyme-linked immunosorbent assay***

While we recruited 101 voluntary participants, sera samples of 99 HBV vaccinated and non-vaccinated study participants were tested in duplicates for the presence of anti-HBs antibodies as a measure of protection against hepatitis B, hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) as indicators of infection. We did not have enough sera to use in the analysis for two of our study participants. The Monolisa HBsAg ULTRA, Anti-HBs PLUS and Anti-HBc PLUS (BIO-RAD, France) were used as per manufacturer’s instructions, respectively. Anti-HBs antibodies were measured in mIU/ml and levels ≥ 10 mIU/ml will be indicative of post-vaccination protection[29,30].

***KIR genotyping***

The Polymerase chain reaction (PCR)-based *KIR* Genotyping SSP Kit (Invitrogen, Brown Deer, WI, United States) was used to detect the presence and absence of *KIR* genes, as per manufacturer’s instructions. Briefly, 25 µl of DNA was used along with the primer sets to amplify the alleles described by the WHO international nomenclature committee (http://www.ebi.ac.uk/ipd/kir/). All amplifications were performed using PX2 thermocycler (ThermoHybrid, United Kingdom) programmed with a 1-min denaturation step at 95 oC, followed by 30 cycles of 94 oC for 20 s, 63 oC for 20 s, and 72 oC for 90 s and finally 4 oC in the thermal cycler. PCR products were gel-purified and visualized under UV transillumination (Sigma, California, United States)[31]. The presence and absence of the following gene loci and variants were tested: 2DL1, 2DL2, 2DL3, 2DL4, 2DL5A, 2DL5B, 2DS1, 2DS2, 2DS3, 2DS5,3DL1, 3DL2, 3DL3, 3DS1, 2DP1, and 3DP1. The variants of the KIR3DP1 pseudogene, KIR3DP\*001/002/004 and KIR3DP1\*003 were also detected in addition to KIR2DS4 variants: 2DS4\*001/002 and 2DS4\*003/007. The frequency of *KIR* was calculated by direct count of the observed phenotype and referred to as observed frequency (OF). In addition, the estimated *KIR* gene frequency (KF) for the putative loci was calculated using the following formula: KF = 1 - √ (1-OF) based on the assumption of Hardy-Weinberg equilibrium[32]. The frequencies of haplotype *A* and *B* were calculated using the following formula: haplotype *A* = (2*n*AA + *n*AB)/2*n* and haplotype *B* = (2*n*BB + *n*AB)/2*n*, where *n*AA, *n*AB and *n*BB are the numbers of individuals with haplotype group AA, AB and BB, respectively and *n* is the total number of individuals[33].

***Statistical analysis***

SPSS 19 was used for statistical analyses. We compared vaccinated and non-vaccinated subjects on each of the *KIR* polymorphism using Chi-square and Fisher-exact test (FET) and report the odds ratio and 95% confidence interval. Similar analyses were conducted for the comparison of protected and non-protected subjects within the vaccinated group. We also examine the relationship between genotypes and the presence or absence of *KIR* genes and the levels of anti-Hbs and *KIR* genes among the vaccinated subjects using Chi-square and FET; for these comparisons, post-hoc tests were conducted only if the omnibus test was significant. We correct for multiple comparisons for post-hoc tests using Bonferroni correction.

**RESULTS**

***Characteristics of study participants***

101 subjects were recruited during March 2010 to December 2011. 39% of the study participants were males and 61% were females. The majority of our study participants were 19-29 years old (40%). Table 1 summarizes the characteristics of the study participants. The majority (75%) held a university undergraduate degree or higher and was employed. During recruitment and when participants were asked about their vaccine status, 50% of our voluntary participants self-reported that they were vaccinated against HBV whereas 25% thought they were not vaccinated and 26% did not know their vaccine status.

In an attempt to confirm the vaccination status of our study participants, Sera samples from 99 participants were tested for the levels of anti-HBs as an indicator of protection (≥ 10 mIU/ml) as defined by the WHO international reference standard[30,34]. This is especially due to the lack of documented dosages of hepatitis B vaccine for many of the study participants as well as lack of knowledge of the vaccination status of many of the study participants. In the analyses thereafter, data on these 99 voluntary subjects are reported; participants with anti-HBs antibodies ≥ 10 mIU/ml will be conisdered vaccinated against hepatitis B. We also tested the sera samples for anti-HBc as a marker of previous infection and for HBs Ag, a marker associated with recent exposure to HBV. All our participants were negative for HBsAg. 74/99 (75%) of our voluntary participants were vaccinated against hepatitis B as judged by the detection of anti-HBs titers; whereas 25% (25/99) were classified as non-vaccinated against hepatitis B. Among the vaccinated participants, 47% (35/74) had anti-HBs titers above 100 mIU/ml, 22% (16/74) with anti-HBs ranging between 10-100 mIU/ml, and 20% (15/74) with values of less than 10 mIU/ml. The time of vaccination (when available) ranged between the years 1999 and 2011 with some participants receiving 2 doses and others receiving 3 or more doses. When we tested for an association between the age groups of our study participants (19-29, 30-39, 40-49, 50-59, 60-69, 70-79 and 80-89) and the concentration of anti-HBs antibodies among vaccinated subjects, a *p* value of 0.047 is detected (FET). 9% (7/74) of the vaccinated participants had undetectable levels of protective antibodies. 5 out of 7 (71%) of the former group were health-care workers; the latter are expected to be continuously monitored for protection against HBV due to the nature of their work. Importantly, anti-HBc was positive in 3% (3/99) of our study participants presenting with anti-HBs levels ranging between 110-1000 mIU/ml. This is associated with protection as a result of natural infection. One of these participants is a vaccinated female nurse whereas the other 2 are non-vaccinated and are not in the health care profession. None of the anti-HBc positive participants were HBsAg positive.

***KIR genotypes and genes frequencies***

We next determined the *KIR* genotypes among the study participants. 44%, 40% and 16% were carriers of AA, AB and BB genotypes, respectively with a 1.77 A to B ratio. The genotype was classified as B if any of the following genes was detected: 2DL2, 2DL5, 3DS1, 2DS1, 2DS2, 2DS3, and 2DS5. If none of these was detected, the genotype is considered as AA. Similarly, if none of the A haplotypes was detected; the genotype is classified as BB. 77%, 75% and 69% of the AA, AB and BB carriers were vaccinated, respectively. There was no significant difference between the expression of AA, AB and BB among vaccinated and non-vaccinated participants (FET, *p* = 0.784). Similarly, we did not detect any significant difference when we compared the frequencies of KIR genotypes among the vaccinated with anti-HBs levels less than 10, 10-100 and above 100 mIU/ml. We did not detect any difference between the frequencies of *KIR* genotypes among vaccinated participants with protective and non-protective levels of anti-HBs (FET, *p* = 0.865).

We divided the *KIR* genes expressed in our study participants into inhibitory, non-inhibitory (or activating) and those encoding inhibitory and activating signals as previously described[13]. The inhibitory KIR2DL1, KIR2DL4, KIR3DL1, KIR3DL2, and KIR3DL were detected in more than 95% whereas KIR2DL2, KIR2DL3, KIR2DL5 (KR2DL5A and KIR2DL5B) were expressed in 56%, 84%, and 42 % (25% and 29%) of participants, respectively (Table 2). The OF of the activating KIR genes ranged between 35% and 55% except for KIR2DS4, detected in 95% of the study participants (40.6% 2DS4\*001/002; 82.2% 2DS4\*003/007). KIR2DP1 pseudogene was detected in 99% of our participants whereas KIR3DP\*001/02/04 and KIR3DP1\*003 had frequencies of 17% and 100%, respectively. The corresponding estimated frequencies of each *KIR* genes followed the same trend or order as the OF data.

For this analysis and thereafter, we studied the genes with enough variability, particularly 2DL2, 2DL3, 2DL5 (2DL5A, 2DL5B), 2DS1, 2DS2, 2DS3, 2DS4 (and its variants), 2DS5, 3DS1 and the pseudogene 3DP1\*001/002/004 (Table 2). We report the lack of significant difference in the frequency of KIR genes among vaccinated and non-vaccinated participants (Table 3). Moreover, there was no significant difference in the frequency of these genes among participants protected against HBV and those that are not protected against HBV , as judged by their level on anti-HBs antibodies (Table 4).

We performed similar analyses to determine the relationship between AA, AB, BB genotypes and the expression of *KIR* genes showing enough variability (Table 5). 2DL2 (χ2, *p* = 0.00), 2DL3 (χ2, *p* = 0.00), 2DS2 (χ2, *p* = 0.00), 2DS3 (χ2, *p* = 0.00), 2DL5B FET, (*p* = 0.00), and 3DP1001/002/004 ( FET, *p* = 0.006 ) were found to be significantly different between genotypes but not 2DS1(χ2, *p* = 0.749) , 2DL5A (FET, *p* = 0.782), 2DS4\*001/002 (χ2, *p* = 0.621), 2DS4\*003/007 (FET, *p* = 0.392) and 3DS1 (χ2, *p* = 0.948). For those genes showing significant difference among the AA, AB and BB genotypes, we computed posthoc comparisons. These differences still hold significant between subgroups except for 2DL5B, 2DS2, 2DS3 and 3DP1\*001/002/004 between genotypes AB and BB.

***KIR genes frequencies among vaccinated and non-vaccinated participants***

To evaluate the role of *KIR* genes in protection against or susceptibility to HBV, we next compared the frequency of *KIR* genes among HBV-vaccinated and non-vaccinated participants (as judged by the level of anti-HBs antibodies). Depending on their anti-HBs levels that we tested for in this study, participants vaccinated against hepatitis B were divided into 4 groups: group I > 100 mIU/ml, group II 10-100 mIU/ml, group III < 10 mIU/ml and group IV with undetectable levels of protective antibodies. The frequency of only KIR2DL5B is significantly different among these categories (FET, *p* = 0.0263) (Table 6). When we performed post-hoc comparisons between these groups, we detected no significant difference in the expression of KIR2DL5B. These groups of vaccinated participants were also similar in relation to the expression of AA, AB and BB genotypes (FET, *p* = 0.669).

We identified 3 participants testing positive for anti-HBc with anti-HBs levels higher than 100 mIU/ml. Two out of three of these participants were non-vaccinated and thus we believe they are protected as a result of natural infection. The third participant was vaccinated against HBV. 2/3 (66.7%) of these participants carry the AB genotype and one participant is AA positive. The three participants expressed 2DL1, 2DL3, 3DL1, 3DL2, 3DL3 (inhibitory), activating genes (2DS3, 2DS4\*001/002 variant) and both inhibitory and activating genes (2DL4, 2DP1 and 3DP1\*003). These subjects did not express 2DL5A or 3DP1\*001/002/004. We did not test for the presence of HBV DNA among these participants. We did not find any significant relationship between the genotype of these participants and the expression of *KIR* genes nor between the *KIR* genes and the susceptibility to natural or breakthrough infection.

**DISCUSSION**

The administration of hepatitis B vaccine in infancy is 95% effective and correlates with long-term protection[8,35,36]. However, vaccine failure has been reported in 5% of hepatitis B-vaccinated persons; moreover, breakthrough infection has also been reported following vaccination with hepatitis B vaccine[37]. The increase in circulating NK cells, major players in the innate immune system and regulators of the virus-specific T cell responses through their cross-talk with dendritic cells and T cells[38-40], was suggested to contribute to HBV viral control[41].The impact of genetic regulation on immune responses following vaccinations has been previously reported[19,24]. This evidence prompted us to explore the potential role of KIR following hepatitis B vaccination.In Lebanon, hepatitis B vaccine is offered as part of the immunization program early in childhood as per the the WHO guidelines. In this study, 69% of the vaccinated participants retained more than 10 mIU/ml of anti-HBs antibodies and hence are immune to HBV infection; whereas 30% are susceptible to the latter due to either undetectable levels of antibodies or levels below 10mIU/ml. We do not have data on the time of vaccination of these participants to reflect on the duration of the retention or the loss of the immune response post-vaccination. We report the lack of significant association between the number of vaccine dosages (when vaccine dosage is available) and the titer of antibodies among vaccinated participants. Recent reports show that multiple immunization against hepatitis B are inefficient at mounting antibody responses[42], while others suggest that immunization against hepatitis at infancy is associated with a seroprotective response to a challenge dose of vaccine with extended duration of protection through adolescent years[43]. We cannot suggest similar trends from our results due to the lack of data on the time of vaccination, the age at vaccination as well as the number of dosages administered for many participants.

Anti- HBc antibodies, indicators of HBV infection, were detected in 3 participants (3%) in the absence of HBsAg with one being a nurse suspected of being exposed to HBV at the work place. This might suggest a “breakthrough” infection occurring following vaccination against hepatitis B; this is suggested since health care workers are regularly monitored for protective levels of anti-HBs antibodies. However, due to the lack of data on the timing of vaccination and/or infection of this participant, we cannot confirm whether exposure to HBV has occurred before or after vaccination. The other 2 participants are non-vaccinated and are protected with high levels of anti-HBs antibodies as a result of natural infection. We do not have data pertaining to the time of infection following vaccination; moreover, we did not perform HBV DNA testing. Health care workers with undetectable anti-HBsAg levels detected in our study are clearly susceptible to HBV infection and consequently in need for booster vaccination to induce an anamnestic response in order to prevent acute disease and carrier state.

While hepatitis B vaccine booster doses are not currently recommended following vaccination, a better understanding of the correlates of long-term immunity is needed. This is critical especially since several studies show that vaccines with anti-HBs levels of 10-99 mIU/ml achieved following primary vaccination are less likely to produce an anamnestic response following a booster HBV vaccine as compared to those with anti-HBs ≥ 100 mIU/ml[35,44]. NK cells play a major role in the innate immune system as first line of defense and in the regulation of the virus-specific T cell responses through their cross-talk with dendritic cells and T cells[38-40]. Moreover, NK cells are suggested to contribute to HBV control[41]. Our data show that genotypes with 11 *KIR* genes were most prevalent with AA genotype being more frequent among the study participants. The inhibitory KIR genes were more frequent among our study participants than the activating genes, which is in agreement with a finding associated with A haplotype being present in higher numbers in inhibitory *KIR* genes[39].

KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1\*003 were present in every participant. This is expected since these are framework genes. The frequency of KIR2DL5B is the only significantly different gene among the vaccinated participants with different anti-HBs antibodies titer. The role of KIR2DL5, expressed at frequencies ranging between 26 and 86% in all human populations, is not completely understood[45]. The ligand of KIR2DL5 is also still unknown.

A number of limitations exist and these include the lack of data on the time of vaccination and corresponding age at time of vaccination of the study participants and more importantly the small sample size. Our sample size is powered to detect medium to large effect sizes when some of the ES for group differences are small. However, medium to large effect sizes are the ES where group differences have more clinical significance, which we are powered to detect. Consequently, the clear impact that KIR genes have on susceptibility to acquiring hepatitis B or protection against the infection cannot be addressed in these small groups.

To our knowledge, this is the first study screening for the possible role of *KIR* genes among individuals vaccinated against HBV. While studies have shown the association between gene variants and immune responses to a variety of vaccines, little is known about the strength and the sustainability of antibody responses following vaccination against HBV in relation to expression of *KIR* genes. Our results are useful to design larger studies to better elucidate the role of KIR in susceptibility or long-term protection against HBV as well as other diseases.

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**COMMENTS**

***Background***

Killer immunoglobulin receptors (*KIR*) genes encode receptors expressed on the surface of natural killer (NK) cells. The literature has described the relationship between KIRs and differential responses in many disease conditions, specifically human immunodeficiency virus (HIV) and hepatitis C virus (HCV). We thought to explore the role of *KIR* genes in response to hepatitis B vaccine in a cohort of Lebanese adults.

***Research frontiers***

This study aims at elucidating the possible role of genetic factors such as KIRs in the regulation of post-vaccine immune responses specifically following hepatitis B vaccine.

***Applications***

While these sample size is powered to detect medium to large effect sizes, the impact that KIR and HLA have on the susceptibility to acquiring hepatitis B virus (HBV) or protection against the infection cannot be addressed in our sample. Nevertheless, our results are useful to design larger studies to better elucidate the role of KIR in susceptibility or long-term protection against HBV and other diseases.

***Terminology***

Two groups of killer immunoglobulin receptors (KIR) haplotypes are known in humans: A and B. Haplotype A encodes inhibitory receptors and consists of nine genes (3DL3, 2DL3, 2DP1, 2DL1, 3DP1, 2DL4, 3DL1, one activating (2DS4), 3DL2 and 2DL5). Haplotype B carries a variety of gene combinations and encodes more activating receptors as compared to haplotype A (3DL3, 2DS2, 2DL2, 2DL5B (inhibitory), 2DS3, 2DP1, 2DL1, 3DP1, 2DL4, 3DS1, 2DL5A (inhibitory), 2DS5, 2DS1, and 3DL2).

***Peer-review***

This is an interesting study aiming to screen for a possible role of *KIR* genes expression and antibody response following hepatitis B vaccination. The major point of this manuscript is that there is no significant association between the frequency of *KIR* genes and anti-HBs antibodies was detected. Although it is negative result, it could be an indicant for understanding the role of *KIR* loci in response to HB vaccine.

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**Table 1 Demographics and characteristics of all participants**

|  |  |  |
| --- | --- | --- |
|  | ***n*** | **Percentage (%)** |
| Gender (*n* = 101) |  |  |
| Male | 39 | 38.6 |
| Female | 62 | 61.4 |
| Age (yr) (*n* = 95) |  |  |
| 19-29 | 38 | 40.0 |
| 30-39 | 19 | 20.0 |
| 40-49 | 17 | 17.9 |
| 50-59 | 11 | 11.6 |
| 60-69 | 6 | 6.3 |
| 70-79 | 2 | 2.1 |
| 80-89 | 2 | 2.1 |
| Education (*n* = 99) |  |  |
| Illiterate | 3 | 3.0 |
| Primary School Education | 3 | 3.0 |
| Secondary School Graduate | 9 | 9.1 |
| High School Education | 10 | 10.1 |
| University Undergraduate Level | 50 | 50.5 |
| Others | 24 | 24.2 |
| Occupation (*n* = 99) |  |  |
| Student | 15 | 15.2 |
| Employed | 74 | 74.7 |
| Unemployed | 7 | 7.1 |
| Retired | 3 | 3 |
|  |  |  |

**Table 2 The observed (OF) and estimated (KLF) *KIR* genes frequencies in the study participants**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Inhibitory KIR**  **\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_** | | | | | | | | **Non-inhibitory KIR**  **\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_** | | | | | | **Pseudogene**  **\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_** | | |
|  | 2DL1 | 2DL2 | 2DL3 | 2DL4 | 2DL5 | 3DL1 | 3DL2 | 3DL3 | 2DS1 | 2DS2 | 2DS3 | 2DS4 | 2DS5 | 3DS1 | 2DP1 | 3DP\* 001/002/004 | 3DP1\* 003 |
| OF | 99 | 56 | 84 | 100 | 42 | 96 | 100 | 100 | 40.6 | 55.4 | 46.5 | 95 | 34.7 | 41.6 | 99 | 17 | 100 |
| KLF | 0.9 | 0.34 | 0.6 | 1 | 0.24 | 0.8 | 1 | 1 | 0.23 | 0.33 | 0.27 | 0.78 | 0.19 | 0.24 | 0.9 | 1 | 1 |

2DL5A, 24.8%; 2DL5B, 28.7%; 2DS4\*001/\*002, 40.6%; 2DS4\*003/007, 82.2%. OF: observed frequency calculated by direct counting; KLF: gene frequency calculated using the formula 1-√ (1-OF).

**Table 3 *KIR* genes frequencies among study participants vaccinated against hepatitis B virus (protected and non-protected by hepatitis B vaccine) *n* (%)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ***KIR* genes** | **anti-HBsAg**  **< 10 mIU/ml**  **(*n* = 22)** | **anti-HBsAg**  **≥ 10 mIU/ml**  **(*n* = 52)** | **Test1** | **OR2 (95%CI)** | ***P* value** |
| 2DL2 | 14 (63.60) | 27 (51.90) | 0.858 | 0.62 (0.22-1.72) | 0.446 |
| 2DL3 | 18 (81.80) | 45 (86.40) | FET | 1.43 (0.37-5.48) | 0.723 |
| 2DL5A | 8 (36.40) | 13 (25.00) | 0.982 | 0.58 (0.20-1.70) | 0.4 |
| 2DL5B | 5 (22.70) | 19 (36.50) | 1.346 | 1.97 (0.62-6.16) | 0.289 |
| 2DS1 | 11 (50.00) | 19 (36.50) | 1.162 | 0.58 (0.21-1.58) | 0.31 |
| 2DS2 | 14 (63.60) | 26 (50.00) | 1.157 | 0.57 (0.21-1.59) | 0.318 |
| 2DS3 | 9 (40.90) | 23 (44.20) | 0.069 | 1.15 (0.42-3.15) | 0.804 |
| 2DS4\*001/002 | 8 (36.40) | 20 (38.40) | 0.029 | 1.09 (0.39-3.072) | 1.00 |
| 2DS4\*003/007 | 19 (86.40) | 41 (78.85) | FET | 0.59 (0.15-2.36) | 0.534 |
| 2DS5 | 10 (45.50) | 17 (32.60) | 1.087 | 0.58 (0.21-1.62) | 0.428 |
| 3DS1 | 11 (50.00) | 21 (40.40) | 0.582 | 0.68 (0.25-1.85) | 0.608 |
| 3DP\*001/002/004 | 6 (27.30) | 9 (17.30) | FET | 0.56 (0.17-1.82) | 0.355 |

We compared the frequencies of *KIR* genes with enough variability between study participants that were vaccinated against hepatitis B virus. The vaccinated participants were divided for this analysis into 2 groups depending on the level of anti-HBs Ag. anti-HBsAg < 10mIU/ml, not protected against hepatitis B virus infection; anti-HBs Ag ≥ 10 mIU/ml, protected against hepatitis B virus infection as a result of vaccination. 1The statistical test performed to compare the frequencies of *KIR* genes expression among vaccinated and non-vaccinated study participants where by FET refers to Fisher’s Exact Test and the rest of the values represent the Pearson Chi-square value. 2OR (95%CI): odds ratio calculation and 95% confidence interval.

**Table 4 *KIR* genes frequencies among study participants vaccinated against hepatitis B virus as compared to non-vaccinated subjects *n* (%)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ***KIR* genes** | **Vaccinated (*n* = 74)** | **Non-vaccinated (*n* = 25)** | **Test1** | **OR2 (95%CI)** | ***P* value** |
| 2DL2 | 41 (55.40) | 15 (60.00) | 0.161 | 0.83(0.33-2.082) | 0.436 |
| 2DL3 | 63 (85.10) | 20 (80.00) | 0.364 | 1.43(0.44-4.62) | 0.374 |
| 2DL5A | 21 (28.40) | 4 (16.00) | 1.517 | 2.08(0.64-6.79) | 0.168 |
| 2DL5B | 24 (32.40) | 5 (20.00) | 1.395 | 1.92(0.64-5.73) | 0.178 |
| 2DS1 | 30 (40.50) | 10 (40.00) | 0.002 | 1.02(0.41-2.58) | 0.577 |
| 2DS2 | 40 (54.10) | 15 (60.00) | 0.268 | 0.78(0.31-1.97) | 0.39 |
| 2DS3 | 32 (43.20) | 14 (56.00) | 1.223 | 0.60(0.24-1.49) | 0.191 |
| 2DS4\*001/002 | 28 (37.80) | 11 (44.00) | 0.297 | 0.77(0.31-1.94) | 0.376 |
| 2DS4\*003/007 | 60 (81.10) | 22 (88.00) | FET | 0.58(0.15-2.23) | 0.324 |
| 2DS5 | 27 (36.50) | 7 (28.00) | 0.597 | 1.47(0.55-3.99) | 0.302 |
| 3DS1 | 32 (43.20) | 9 (36.00) | 0.246 | 1.35(0.53-3.46) | 0.401 |
| 3DP\*001/002/004 | 15 (20.30) | 2 (8.00) | FET | 2.92(1.62-13.80) | 0.134 |

We compared the frequencies of *KIR* genes with enough variability between study participants that were vaccinated against hepatitis B virus and those that were not. 1The statistical test performed to compare the frequencies of *KIR* genes expression among vaccinated and non-vaccinated study participants where by FET refers to Fisher’s Exact Test and the rest of the values represent the Pearson Chi-Square value. 2OR (95%CI): odds ratio calculation and 95% confidence interval.

**Table 5 The relationship between AA, AB and BB genotypes and the expression of *KIR* genes among the study participants *n* (%)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ***KIR* genes** | **AA (*n* = 44)** | **AB (*n* = 41)** | **BB (*n* = 16)** | **Test1** | ***P* value** |
| 2DL2 | 0 (0.00) | 41 (100.00) | 16 (100.00) | 101 | 0.00a |
| 2DL3 | 44 (100.00) | 41 (100.00) | 0 (0.00) | 101 | 0.00a |
| 2DL5A | 10 (22.70) | 10 (22.70) | 5 (31.25) | FET | 0.782 |
| 2DL5B | 1 (2.27) | 20 (24.39) | 8 (50.00) | FET | 0.00a |
| 2DS1 | 16 (36.30) | 18 (43.90) | 7 (43.75) | 0.579 | 0.749 |
| 2DS2 | 0 (0.00) | 40 (97.50) | 16 (100.00) | 97.051 | 0.00a |
| 2DS3 | 3 (6.80) | 30 (73.17) | 14 (87.50) | 50.38 | 0.00a |
| 2DS4\*001/002 | 16 (36.40) | 19 (37.50) | 6 (37.50) | 0.952 | 0.621 |
| 2DS4\*003/007 | 38 (86.40) | 31 (75.60) | 14 (87.50) | FET | 0.392 |
| 2DS5 | 14 (31.80) | 16 (39.00) | 5 (31.30) | 0.584 | 0.747 |
| 3DS1 | 18 (40.90) | 18 (43.90) | 6 (40.00) | 0.107 | 0.948 |
| 3DP\*001/002/004 | 2 (4.50) | 10 (24.40) | 5 (31.30) | FET | 0.0006a |

1The statistical test performed to determine the relationship between AA, AB and BB genotypes and the expression of KIR genes among vaccinated and non-vaccinated study participants. FET refers to the Fisher’s Exact Test and the rest of the values represent the Pearson Chi-square value. a*p* < 0.05, significant.

**Table 6 *KIR* genes expression and levels of anti-HBs among the vaccinated study participants *n* (%)**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| ***KIR* genes** | **Group I1 (*n* = 36)** | **Group II2 (*n* = 16)** | **Group III3 (*n* = 15)** | **Group IV4 (*n* = 7)** | | **Test5** | ***P* value** |
| 2DL2 | 17 (47.20) | 10 (62.50) | 11 (73.30) | 3 (42.90) | | FET | 0.362 |
| 2DL3 | 31 (86.10) | 14 (87.50) | 12 (80.00) | 6 (85.70) | | FET | 0.824 |
| 2DL5A | 9 (25.00) | 4 (25.00) | 4 (26.70) | 4 (57.10) | | FET | 0.987 |
| 2DL5B | 9 (25.00) | 10 (62.50) | 4 (26.70) | 1 (14.30) | | FET | 0.0236 |
| 2DS1 | 11 (30.60) | 8 (50.00) | 7 (46.70) | 4 (57.10) | | FET | 0.530 |
| 2DS2 | 16 (44.40) | 10 (62.50) | 11 (73.30) | 3 (42.90) | | FET | 0.270 |
| 2DS3 | 12 (33.30) | 11 (68.80) | 8 (53.30) | 1 (14.30) | | 5.01 | 0.060 |
| 2DS4\*001/002 | 15 (41.70) | 5 (31.30) | 6 (40.00) | 2 (28.60) | | 0.568 | 0.920 |
| 2DS4\*003/007 | 28 (77.80) | 13 (81.30) | 13 (86.70) | 6 (85.70) | | FET | 0.860 |
| 2DS5 | 11 (30.60) | 6 (37.50) | 6 (40.00) | 3 (42.90) | | 0.846 | 0.850 |
| 3DS1 | 14 (38.90) | 7 (43.80) | 7 (46.70) | 6 (85.70) | | FET | 0.960 |
| 2DL4 | 36 (100.00) | 16 (100.00) | 15 (100.00) | 4 | 57.10 | 0.258 | 0.970 |
| 3DP\*001/002/004 | 8 (22.20) | 1 (6.30) | 4 (26.70) | 1 | 14.30 | FET | 0.420 |

1Group I: vaccinated participants and anti-HBs titers > 100 mIU/ml; 2Group II: vaccinated participants and anti-HBs titers 10-100 mIU/ml; 3Group III: vaccinated participants and anti-HBs titers 0.1-9.99 mIU/ml; 4Group IV: vaccinated participants and anti-HBs titers = 0 mIU/ml; 5The statistical test performed to compare the frequencies of *KIR* genes expression among the vaccinated study participants where by FET refers to Fisher’s Exact Test and the rest of the values represent the Pearson Chi-Square value; 6Significant difference of KIR2DL5B expression among vaccinated study participants (*p* < 0.05).