Clinical implication of soluble hla-g and immunogenetic influence of hla-g +3142g/c polymorphism in breast cancer development among patients attending ORCI in Tanzania

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Clinical Implication of Soluble HLA-G and Immunogenetic Influence of HLA-G +3142G/C Polymorphism in Breast Cancer Development among Patients attending ORCI in Tanzania

By

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A Dissertation Submitted in (partial) Fulfillment of the Requirements for the Degree of Master of Science (Biochemistry) of

Muhimbili University of Health and Allied Sciences

October, 2019

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled "***Clinical Implication of Soluble HLA-G and Immunogenetic Influence of HLA-G +3142G/C Polymorphism in Breast Cancer Development among Patients attending ORCI in Tanzania*****"**, in (Partial) fulfillment of the requirements for the degree of Master Science in Biochemistry of Muhimbili University of Health and Allied Sciences.

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DECLARATION AND COPYRIGHT

I, **Ismael Chatita Adolf**, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award

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ABSTRACT

Background: Breast cancer is among the most prevalent cancers in Tanzania. Cancer arises from aberration in regulatory systems controlling cell cycle. Many altered (tumor) cells are kept in check by the immune system due to their altered expression of cell surface molecules. Yet some tumor cells do manage to escape immune recognition and develop into malignancy, and immunosuppressive HLA-G is thought to be expressed more by tumor cells and enable them achieve this immune escape. Soluble Human Leukocyte Antigen-G (sHLA-G) has been closely associated with diagnosis and prognosis in many types of human cancer.Moreover, +3142G/C polymorphism (rs1063320) in HLA-G gene affects its expression, and G is considered to be a protective mutant allele associated with less expression of HLA-G. The implication of HLA-G in cancer development has been reported in different cancers and in different populations. But, its implication in Tanzanian population has not yet been investigated.

Aim: The goal of this study was to investigate the extent of soluble HLA-G level and the frequencies distribution of +3142G/C genetic variants affecting its expression, and their associations with cancer development among breast cancer patients attending ORCI in Tanzania.

Method: This was a case-control study, where 75 breast cancer patients and 84 normal healthy women were recruited. Most patients (81.3%) had undergone mastectomy and were under adjuvant therapy. The remaining patients, though non-mastectomized were also under treatment regimen. Plasma level of soluble HLA-G protein was determined by ELISA. The genotyping of HLA-G +3142G/C polymorphism was determined by LightSNiP typing assay using quantitative Real-Time PCR**.**

Results: The frequencies distribution of +3142C/G genotypes and alleles were relatively similar between patients and controls (Genotype, p=0.537; Alleles, p=0.650). The protective genotype GG was weakly associated with breast cancer: OR=1.26 (CI 95% 0.6-2.65). The level of sHLA-G molecule in breast cancer patients was significantly lower than that in normal healthy women ($p<0.001$), with an area under the receiver operating characteristics (ROC) curve of 0.697 (CI 95%=0.619-0.767, *p<0.001*). No significant associations of sHLA-G level were found with respect to metastatic and receptors expression (ER, PR and HER2) status. However, sHLA-G level was significantly higher in non-mastectomized group compared to mastectomized one (p=0.017).

Conclusion: The results show low levels of sHLA-G and poor association of +3142G/C SNP with breast cancer among Tanzanian breast cancer patients undertaking neoadjuvant and adjuvant therapies. Medical interventions are more likely to have lowered sHLA-G in these breast cancer patients. More studies with increased sample size including patients with early stage breast cancer and controlling medical interventions are recommended to reach a clear conclusion in order to provide evidence in further support of the application of sHLA-G as a biomarker for breast cancer diagnosis and monitoring of breast cancer therapy in Tanzania.

Key words: Breast cancer, soluble HLA-G, +3142G/C HLA-G, Diagnosis, Mastectomy, Tanzania.

ABSTRACT (KISWAHILI)

Utangulizi: Saratani ya matiti ni mojawapo ya saratani zilizoshamiri kwa wingi hapa Tanzania. Saratani huwa inatokea kutokana na kasoro inayojitokeza katika kuratibu ukuaji na uzalishaji wa chembe hai. Chembe hai nyingi zenye hitilafu (chembe hai za saratani) huwa zinatambuliwa na kuondolewa na mfumo wa kinga mwilini kabla hazijazaliana zaidi na kutengeneza uvimbe. Hata hivyo chembe hai hizo zenye hitilafu huwa hatimaye zinafanikiwa kukua na kutengeneza uvimbe, na molekyuli iitwayo HLA-G inasemekana kuzalishwa kwa wingi na chembe hai za saratani ili kudhoofisha mfumo wa kinga kuweza kuzitambua na kuziondoa hizi chembe hai za saratani. HLA-G iliyoko kwenye damu (sHLA-G) imekuwa ikiambatanishwa na uchunguzi na ubashiri wa saratani mbalimbali za binadamu. Asili ya sehemu ya vinasaba (+3142G/C) inasemekana kuathiri utengezezwaji wa HLA-G. Asili yenye G katika sehemu hiyo inasemekana kuwa na kinga dhidi ya saratani na inahusiana na uzalishwaji mdogo wa hii molekyuli (HLA-G). Namna HLA-G inavyohusika katika ukuaji wa saratani imewasilishwa katika machapisho mbalimbali yaliyotokana na tafiti katika jamii mbalimbali. Lakini, uhusikaji wake katika jamii ya watanzania haujawahi chunguzwa.

Lengo: Lengo kuu la utafiti huu ilikuwa ni kutabainisha namna kiasi cha HLA-G kilichoko kwenye damu na asili ya sehemu ya vinasaba (+3142G/C) inayoathiri uzalishwaji wake vinavyohusiana na ukuaji wa saratani ya matiti katika wagonjwa wa saratai ya matiti walioko ORCI.

Njia: Utafit huu ulilinganisha kundi la wangonjwa wenye saratani ya matati na kundi la wanawake wasiyo na saratani yoyote. Jumla ya wagonjwa 75 na wanawake wasiyo wagonjwa 84 walihusika katika utafiti huu. Wagonjwa wengi (81.3%) walikuwa wameshafanyiwa oparesheni ya matiti kwa ajili ya saratani. Kiasi cha sHLA-G kilichoko kwenye damu kilitabainishwa kwa kutumia ujuzi uitwao ELISA. Pia asili ya vinasaba (+3142G/C) ilitambuliwa kwa kutumia ujuzi uitwao “LightSNiP typing”.

Matokeo: Hakukuwa na utofauti mkubwa katika asili ya sehemu ya vinasaba (+3142G/C) kati ya wagonjwa wa saratani ya matiti na wanawake wasio wagonjwa. Asili ya vinasaba yenye G katika sehemu hiyo (+3142G) inayosemekana kuwa na kinga dhidi ya saratani ilikuwa na uhusiano hafifu na saratani ya matiti. Kiasi cha sHLA-G kilikuwa kidogo sana ukilinganisha na kiasi chake kilichokutwa katika wanawake wasio wagonjwa. Pia, kiasi cha sHLA-G kilikuwa

hakihusiani na kusambaa kwa saratani wala utengezezwaji wa vipokezi vya chembe hai (PR, ER na HER2). Hata hivyo, kiasi cha sHLA-G kilikuwa kidogo sana katika wagonjwa waliofanyiwa oparesheni ya matiti kwa ajili ya saratani ukilinganisha na wagonjwa ambao walikuwa hawajafanyiwa oparesheni hiyo.

Hitimisho: Matokeo ya utafiti huu yanaoneshe kiasi kidogo cha sHLA-G, na uhusiano hafifu wa asili ya sehemu ya vinasaba (+3142G/C) na saratani ya matiti katika wagonjwa wa saratani ya matiti wa kitanzania. Matibabu waliyokuwa wameyapata na kuandelea kupata wagonjwa hawa yanaweza yakawa yamehusika kwa kiasi kikubwa kushusha kiasi chao cha sHLA-G. Tafiti nyingine zaidi zenye kujumuisha sampuli kubwa zaidi na wagonjwa wenye saratani iliyo bado katika steji za mwanzoni na ambao hawajapata matibabu sana, zinahimizwa sana ili kufikia hitimisho lisilo na chembembe za ukinzani ili kutoa ushahidi wa kuunga mkono zaidi matumizi ya sHLA-G kama kiashiria cha uchunguzi wa saratani na cha kutambua maendeleo ya wagonjwa wanaopata matibabu ya saratani ya matiti.

Maneno muhimu: Breast cancer, soluble HLA-G, +3142G/C HLA-G, Diagnosis, Mastectomy, Tanzania.

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ABBREVIATIONS

3’UTR: 3’ Untranslated Region

APC: Antigen Presenting Cells

AU-ROC: Area Under Receiver Operating Characteristic

cAMP: Cyclic Adenosine monophosphate

CD: Cluster of Differentiation

CdK: Cyclin dependent Kinase

CTL: Cytotoxic T Lymphocyte

DC: Dendritic Cell

DNMT: DNA Methyl Transferase

EBV: Epstein Barr Virus

ELISA: Enzyme-Linked Immunosorbent Assay

FasL: First apoptosis signal Ligand

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

HHV: Human Herpes Virus

HIF: Hypoxia Induced Factor

HLA: Human Leukocyte Antigen

HPV: Human Papilloma Virus

HRE: Hypoxia Response Element

HTL: Helper T Lymphocytes

IFN: Interferon

IL: Interleukin

ILT: Immunoglobulin like Transcript

Ins/Del (InDel): Insertion/deletion

ISRE: Interferon Sequence Response Element

ITIM: Immunoreceptor Tyrosine-based Inhibitory Motif

KIR: Killer Immunoglobulin like Receptor

MDSC: Myeloid Derived Suppressor Cells

MFS: Metastatic free survival

MHC: Major Histocompatibility Complex

mHLA-G: membrane bound HLA-G

miRNA: micro RNA

NK: Natural killer

ORCI: Ocean Road Cancer Institute

PBMC: Peripheral Blood Mononuclear Cells

PD-L: Programmed death ligand

RAG: Recombination Activating Gene

RRE: Ras Response Element

rs: reference sequence

sHLA-G: Soluble HLA-G

shRNA: short hairpin RNA

$S_{N:}$ Sensitivity

SNP: Single Nucleotide Polymorphism

$S_{P:}$ Specificity

TATAs: Tumor Associated Transplantation Antigens

Th: Helper T cell

TSTAs: Tumor Specific Transplantation Antigens

URR: Upstream Regulatory Region

VIA: Visual Inspection with Acetic Acid

β2M: β-2-microglobulin

DEFINITIONS OF KEY TERMS

Carcinogenesis/Tumorigenesis/Oncogenesis: The process by which normal cells develop into cancer cells

HLA-G: One of non-classical Human Leukocyte Antigen class I molecule thought to have suppressive effect on immune cells functions

Immunoediting: A dynamic interaction between tumor cells and immune cells that impacts shift in nature of immune response to tumor cells ultimately leading to cancer progression

Immunogenetics: The study of genetic basis of immune system response

Immunosurveillance: The act by which immune cells hunt for and recognize pathogens and altered cells in the body

Odd ratio: A measure of association between a given exposure and disease outcome representing the odds that a disease will occur provided a particular exposure is present compared to the odds of disease occurring in the absence of that exposure

Plasma: Liquid part of blood obtained after centrifugation without prior blood clotting

Polymorphism: Existence of variation at a specific position of a given nucleic acid or protein sequence in the population

Prognosis: An opinion, based on medical experience of the likely course of medical condition

Proto-oncogenes: The genes which encode proteins that drive or enhance the cell to divide

ROC: A curve of sensitivity against (1-specificity) that is used to determine the diagnostic performance of a variable

Sensitivity: The probability of a diagnostic tool to detect a disease given that the disease is truly present

Serum: The liquid part of blood obtained after coagulation

SNP: The existence of nucleotide variation at one specific nucleotide position of a gene (Single nucleotide polymorphism)

Specificity: The probability of a diagnostic tool to miss a disease given that the disease is truly absent

Tumor stage: The extent by which solid cancer has grown and spread in the body

Tumor-suppressor genes: The genes which encodes for proteins that downregulate the cellular division and proliferation.

1.0. INTRODUCTION

1.1. Background

Cancer remains to be a global problem, affecting both developed and less developed countries. Breast cancer is the second cause of cancer death after lung cancer among women in developed countries, but the most leading cause of cancer death in developing countries (1). In sub-Sahara Africa, cervical cancer is the most prevalent and leading cause of cancer death among women followed by breast cancer (2). In East Africa, Tanzania bears a highest burden of cervical cancer (3).

While the overall incidence of cancer is as twice in developed as in developing countries, the mortality rate is higher in developing countries than that in developed countries (1). This ironic difference has been attributed, in developing countries to late cancer diagnosis, poor available treatment and bearing a burden of highly fatal cancer types (1). Most breast cancer patients in Tanzania are diagnosed at late stages (4), and almost half of patients diagnosed with breast cancer die from this disease (5). The trend shows that cancer incidence is shifting from developed countries to less developed countries (1).

Cancer pathogenesis involves the expression of some tumor-associated and tumor-specific molecules which can trigger the immune response to them (6,7). The immune cells recognize these altered cells and exert cytotoxic effect to them. The notable cytotoxic immune cells are CD8+ T cells (Cytotoxic T lymphocytes) and Natural killer (NK) cells. There is some emerging evidence however indicating the additional cytotoxic function to helper function of CD4+ T cells (8).

Despite this impressive function of immune cells to recognize and destroy cancerous cells, tumors normally manage to develop into malignancy by utilizing some escape mechanisms from immune system. The microenvironments created in course of tumorigenesis and some molecules expressed by tumor cells tend to suppress immune cells' functions (9–14). The notable molecule thought to be expressed by various tumor cells with immunosuppressive effect is Human Leukocyte Antigen G (HLA-G) (15–20).

HLA-G is a non-classical HLA class I molecule which is restrictively expressed by cells in immune privileged tissues under normal physiological condition (21). However, its expression

may be upregulated in other cells under certain physiological or pathological conditions. In pregnant women, the trophoblast cells existing at maternal-fetal interface express high level of HLA-G which physiologically protect semi-allogeneic fetus against potential maternal immune system attack (18). Some pathological conditions triggering high level expression of HLA-G include tumorigenesis, chronic inflammatory diseases and some viral infections (22).

The HLA-G is less polymorphic in most populations. It has seven isoforms produced as a result of alternative splicing of its mRNA. Four isoforms are membrane bound while the other three are secretory ones (23). HLA-G interacts with inhibitory receptors on immune cells such as immunoglobulin like transcript (ILT) and killer cell immunoglobulin like receptors (KIR) exerting their immunosuppression effects, such as inhibiting cytotoxic ability and induction of apoptosis (24–26).

Because of its tumorigenesis-induced expression, HLA-G is thought to be a potential biomarker for cancer progression and discrimination between cancer patients and healthy individuals. Soluble HLA-G (sHLA-G) has been extensively studied in context of clinicopathological status of different carcinomas (27–33), and its power to discriminate cancer patients from normal healthy individuals has been demonstrated (30,33–37). Also, the decrease in sHLA-G level in cancer patients has been observed in response to surgery followed by adjuvant therapy, making this molecule a potential candidate for prognostic monitoring of cancer response to treatment (38).

Some controversies however do exist on whether this molecule is significantly implicated in cancer pathogenesis. Some studies have reported low or no expression of HLA-G in tumor tissues (39). Others found the comparable levels of sHLA-G between cancer patients and healthy controls (40) while others reported lack of association between sHLA-G level and cancer progression (37). All these findings discourage the relevance of HLA-G suggested by other studies in carcinogenesis. However, very few similar studies have been conducted in African ethnic groups, and this is the first study to investigate the utility of sHLA-G in defining breast cancer in Tanzanian population and its diagnostic potential.

There is good number of researches showing the influence of genetic configuration on the expression of HLA-G. There are some polymorphisms in the non-coding regions of HLA-G which seem to affect the expression of HLA-G by affecting the affinity of regulatory sequence to

transcription factors and stability of transcribed mRNA (41–45). Because HLA-G expression suppresses the immune response to tumor cells, studying these polymorphisms can potentially reveal their association with cancer susceptibility.

HLA-G 3' UTR 3142G/C polymorphism has been studied in different populations worldwide with respect to risk for cancer development, but with very limited studies in African ethnic groups. The results are however somehow conflicting, with some researchers reporting some positive associations (46) while others reporting no association (47). It is therefore important to study about these polymorphisms in different African ethnic groups, particularly in Tanzania so as to enlighten our insights on the potential influence of these polymorphisms on HLA-G expression and susceptibility to common prevalent cancer types such as breast cancer.

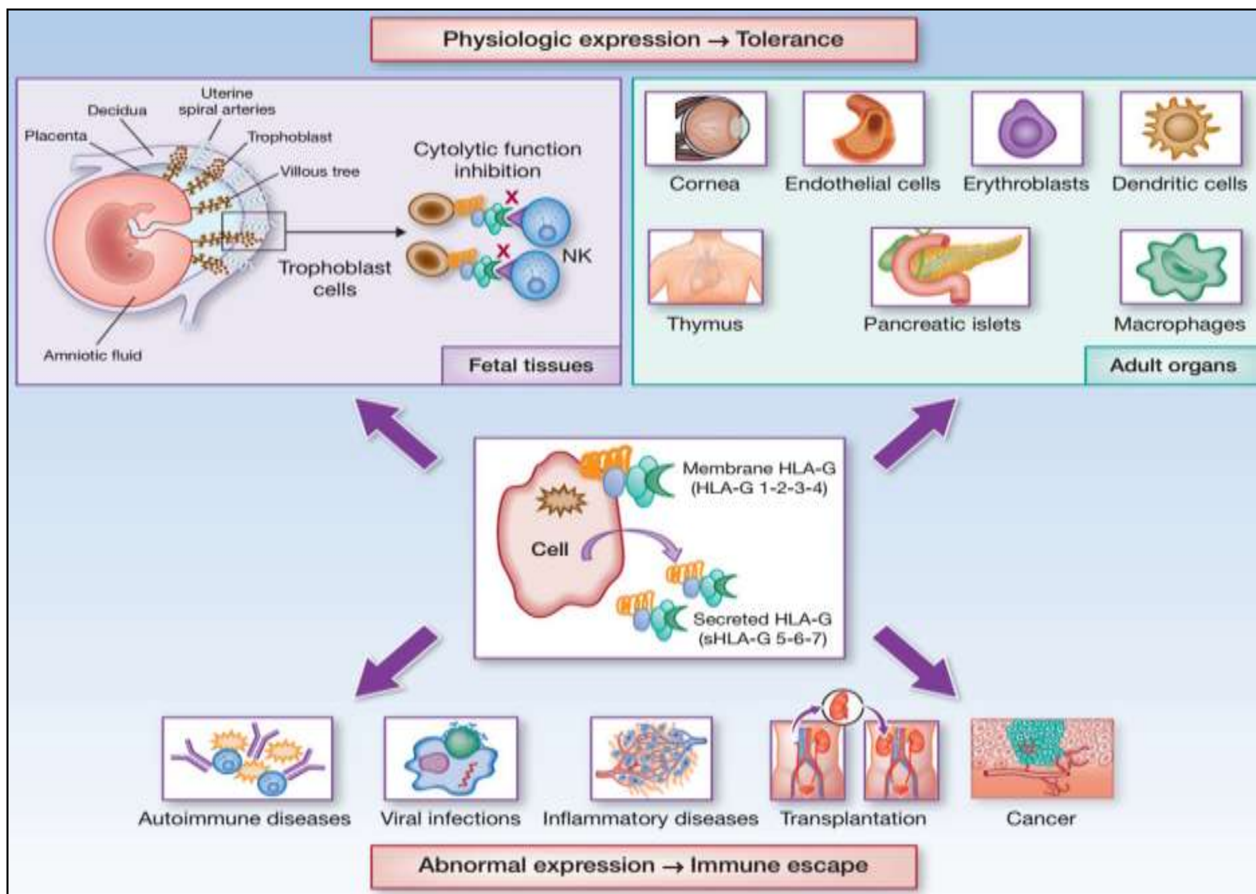


Figure 1: *HLA-G and Immunotolerance (Source: Curigliano et al., 2013)* (48).

At normal physiological conditions, HLA-G is more expressed in immune privileged sites such as cornea, pancreatic islets, endothelial cells, thymus, erythroblasts and in hematopoietic cell lineage. Its expression is altered or deregulated during pregnancy, pathological conditions such as autoimmune diseases viral infections, inflammatory diseases and cancer, and transplantation.

1.2. Statement of the Problem

Breast cancer is the most prevalent cancer type among women world-wide (1). In sub-Sahara Africa, cervical cancer is the leading most common cancer among women, followed by breast cancer (1). In developing countries like Tanzania, half of patients diagnosed with breast cancer die (5). The high cancer mortality rate in developing countries is largely attributed to late presentation of patients to medical care, where most of them get diagnosed when cancer is already in advanced stages (4) with poor response to treatment.

To be able to diagnose breast cancer in its very early stages in most women, screening programs are of paramount importance. Currently used screening method for breast cancer relies on mammography, which is recommended for screening in women aged between 50 to 74 biennially (49). It is not also recommended for pregnant women due to potential effects of the radiation on the developing fetus. Apart from that, common false positive results in younger women and dense-breasted women have been reported (50) and repeatedly exposure to mammography radiation can pose a risk for development of radiation induced breast cancer (51).

Most patients die from cancer due to resistance developed by cancerous cells to different therapeutic remedies such as chemotherapy and radiotherapy. Immunotherapy is considered to be a promising better approach for cancer treatment in the future. The therapy works by activating the host immune cells to recognize and destroy the cancerous cells. However, the efficacy of immunotherapeutic products is lowered due to existence of immunosuppressive environment in and around tumor tissues (17,22,52,53). Overcoming the mechanisms inducing the immunosuppressive environment by tumor cells is pivotal for cancer immunotherapy.

HLA-G seems to be a good target for developing solutions for both diagnostic and therapeutic challenges associated with breast cancer. A number of researches show the increased expression of both membrane-bound and soluble HLA-G in cancer patients along with their diagnostic and prognostic potential (31,35–37,54,55). The immunosuppressive characteristics of HLA-G have also been reported by numerous researchers, recommending it as a good target for immunotherapy based treatment of cancer (8).

There is a scarcity of data on clinical implication of HLA-G and its genetics from African ethnic groups. Very few studies have been done in African countries on HLA-G with respect to cancer development. Particularly in Tanzania, there is no study which have been done to investigate the

association of HLA-G expression and clinicopathological parameters of cancer patients, and the influence of HLA-G gene polymorphisms on HLA-G expression, and hence cancer susceptibility.

1.3. Significance of the Study and Rationale

This study, for the first time has yielded data, based on Tanzanian ethnic groups on how HLA-G expression is implicated in clinical manifestations of breast cancer. Also, the influence of HLA-G +3142G/C polymorphism on HLA-G expression and its association to breast cancer development has been evaluated for the first time in Tanzanian population.

As it has been stipulated in the problem statement, owing to the existing limitations of breast cancer screening methods, there is a significant need for development of relatively cheap and safe diagnostic tools that can be used for screening purposes of this type of cancer. The new diagnostic method might be used independently from or as an adjunct to the existing diagnostic methods useful in breast cancer screening to increase the overall accuracy of the screening process.

This study was meant to contribute to the body of knowledge on the potential utility of soluble HLA-G as a diagnostic biomarker for breast cancer. The determined association between sHLA-G and tumor progression highlights whether sHLA-G can be relied upon as an indicator for breast cancer progression, saving patients from undergoing unnecessary intense and invasive procedure required to collect biopsies for histological examinations. Regarding the immunosuppressive effect of HLA-G which has been reported by many researchers, this study adds to the body of knowledge on whether it is important to target and modify HLA-G for the sake of cancer immunotherapy.

Lastly, due to the reported influence of HLA-G +3142G/C polymorphism on HLA-G expression and the implication of HLA-G in cancer development, the association between this polymorphism and cancer development has been investigated based on Tanzanian population. It was hoped that this polymorphism might serve as a genetic risk factor for breast cancer susceptibility, and hence useful in genetic counseling.

1.4. OBJECTIVES

1.4.1. Broad Objective:

To investigate the extent of soluble HLA-G expression and its underlying immunogenetic influence of HLA-G 3142G/C polymorphisms, and their association with disease onset and progression among patients with breast cancer at Ocean Road Cancer Institute (ORCI) in Tanzania.

1.4.2. Specific Objectives:

i) To determine the extent of soluble HLA-G expression among patients with breast cancer attending at the ORCI in Tanzania.

ii) To determine whether soluble HLA-G expression is associated with tumor progression among breast cancer patients attending at the ORCI in Tanzania.

iii) To determine the individual frequencies of HLA-G 3142G/C alleles and genotypes, and their association with cancer development among breast cancer patients attending at the ORCI in Tanzania.

1.5. Literature Review

1.5.1. Breast Cancer at a Glance

1.5.1.1. Incidence and Risk factors

Breast cancer involves uncontrolled proliferation of cells and subsequent formation of malignancies in mammary gland tissues. It is a neoplastic disorder encompassing heterogeneous cellular and molecular characteristics. The proliferating cells may be derived from epithelium lining milk duct or milk producing lobules or the tissue intervening the two. Sometimes but rarely, the neoplastic cells may originate from stromal cells or epithelial cells lining blood vessels in breast (56).

Breast cancer is the most common cancer among women globally, with an estimated incidence of more than a million new cases per year (57). Over a decade, from 2005 to 2015, the incidence of breast cancer increased from 1.7 million to 2.4 million incident cases (58). The existing evidence is suggestive that the incidence of breast cancer is increasing in Africa. The recent meta-analysis by Adeloye *et al.* showed that the crude incidence of breast cancer in Africa is 24.5 per 100,000 person years (59). Breast cancer is the second leading cause of cancer related deaths among women in developed countries following lung cancer. But, it is the first leading cause of cancer mortality among women in developing countries (1).

Breast cancer is a result of complex interaction of genetic and environmental or life-style related risk factors. Many environmental and life-style related factors have been studied in relation to breast cancer development. These non-genetic factors are summarized in Table 1.

Genetic predisposition accounts for 5 to 10% of all breast cancers. Family history of breast cancer has been reported to be a risk factor of breast cancer. Having first-degree relative suffering/suffered from breast cancer increases the risk of breast cancer. The risk increases with the increase in number of relatives suffering from breast cancer (60). The notable mutation responsible for inheritable breast cancer has been found in breast cancer (BRCA1 and BRCA2) genes, the tumor suppressor genes. The life time risk for a woman with mutation in BRCA1 or BRCA2 gene is up to 65% and 45% respectively. For a woman carrying mutation in both two genes, her life time risk to develop breast cancer increases up to 70% by age of 80 (56).

Although less common than BRCA genes, inheritable mutations in some other genes have also been associated with breast cancer. The inherited mutation in p53 protein encoding gene (TP 53) which causes Fraumeni syndrome has been shown to pose increased risk to breast cancer along with other cancers. Cowden syndrome, caused by inherited mutation in Phosphatase and tensin homolog protein encoding gene (PTEN) is normally accompanied by the increased risk to develop breast and some other tumors. The increased risk of developing lobular breast cancer has been found in people carrying the mutation in Cadherin-1 encoding gene (CDH1), which also causes hereditary diffuse gastric cancer (56).

The Partner-And-Localizer-Of-BRCA2 protein (encoded by PALB2 gene) interacts with BRCA protein, and mutation in this elevates the risk of breast cancer. Mutation in a gene encoding Ataxia Telangiectasia mutated protein (ATM gene) responsible for Ataxia-telangiectasia disorder raises the chance for inheritable forms of breast cancer. Peutz-Jeghers syndrome is caused by inherited mutation in a gene encoding serine/threonine kinase 11 protein (STK11) which is accompanied with higher chance to develop various cancers including breast cancer. The mutation in the checkpoint gene (CHEK2) has also been associated with increased chance to develop breast cancer (56).

1.5.1.2. Types of Breast Cancers

Breast cancers are classified mainly on basis of site and cell of their origin, and whether they have invaded the surrounding tissue. Breast cancer can be classified as carcinoma or sarcoma depending on the cell origin. Breast carcinomas are derived from epithelial cells lining the milk duct (ductal carcinoma) or lobules (lobular carcinoma) of the breast, while breast sarcomas originate from blood vessel cells and myofibroblasts making up stroma of the breast. Breast sarcomas are very rare, and carcinomas are the most commonly found breast cancers (56). Depending on the invasive and other pathological features, breast cancer can further be divided as Non-invasive (In situ), Invasive and Metastatic. The most common non-invasive breast cancer is ductal carcinoma in situ (DCIS). This cancer is confined at the ductal site of its origin without invading the neighboring tissues. Lobular carcinoma in situ (LCIS) is currently not classified as a cancer, but a merely benign tumor. LCIS can only develop into cancer if not treated (56).

Table 1: Non-Genetic Risk factors of Breast Cancer (BC)

Risk Factor	Description	Reference
Exposure to radiation	Previous exposure to chest radiation therapy especially at younger age increases the risk of BC	(61)
Cumulative hormonal exposure	The more lifetime exposure of breast to estrogen and progesterone, the more risk of developing BC. Experiencing more menstrual cycles as a result of early menarche, delayed menopause (i.e. after age 50) and giving birth after age 30 or not giving birth at all increases the lifetime exposure to estrogen and progesterone	(62)
Contraceptives and HRT	These contain estrogen or/and progesterone which pose risk to develop BC	(63)
Excessive alcohol consumption	The risk increases with an increased amount of alcohol consumed	(64)
Increased age	The risk of BC increases with an increase in age. Most BC cases occurs in women above age 50	(65)
Overweight and obesity	Fat tissues make estrogen, especially after menopause. High adiposity increases the level of estrogen which poses the risk of BC	(56)
Tobacco use	Tobacco smoke contains various carcinogenic substances which can cause different cancers, including BC	(66)
Impaired immune system	Immunosuppression caused by drugs and infection raises the risk of developing BC and other cancers	(67)
Nutrition	Higher consumption of fats and reduced consumption of fruits and vegetables increases the risk of BC	(68)
Physical inactivity	Regular physical activity is considered to be protective against BC	(68)
Benign breast conditions	Non-proliferative lesions such as fibrosis, simple cysts, duct ectasia, periductal fibrosis and apocrine and squamous metaplasia may marginally affect the risk of BC	(56)
Gender	BC is very common in women, the incidence in men is very small	(65)

Invasive, also known as infiltrating breast cancer grows beyond the ductal or lobular site of their origin by invading and spreading into neighboring tissues that form stroma. Around 90 to 95% of breast cancer cases are invasive. They can be further grouped as invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) depending on whether they originate from duct or lobule respectively. IDC is the most common, and up to 80% of breast cancer cases have IDC (69).

Metastatic breast cancer manifests when cancer cells have migrated and invaded distant organs. It is also referred to as stage IV or late stage cancer. The common metastatic sites for breast cancer are lymph nodes, lungs, bones, liver and brain (70). Micro-metastases remaining after removal of primary tumors may be responsible for recurrence of cancer.

Some other types of breast cancers are less common. Inflammatory breast cancer (IBC) is invasive and very aggressive breast cancer accounting up to 5% of all cases. Lump is not present in IBC, making it unable to be detected on mammogram. Other uncommon types of breast cancer include Paget disease of the breast, Papillary carcinoma, Phyllodes tumor and angiosarcoma of the breast (56).

Furthermore, breast cancers can be classified based on the expressivity of three receptors; estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth receptor 2 (HER2). This classification is important in dictating the therapeutic options of breast cancer based on the type of receptor expressed (70).

1.5.1.3. Staging and Grading of Breast Cancer

While cancer stage reflects how largely tumor has grown and spread in the body, cancer grade tells how aggressive tumor cells are. The most common approach of staging breast cancer is tumor, node, metastasis (TNM) system, which stages breast cancer into stage 0, stage I, stage II, stage III and stage IV (70). The details are presented in Table 2.

Tumor grading involves examination of morphological features of tumor cells as the proxy for tumor differentiation status. The poorly differentiated tumors have worse prognosis, and are considered to be of high grade. There are two types of tumor grades; histologic grade and nuclear grade. Nuclear grading is restricted to ductal carcinoma in situ (DCIS), and histologic grade is the most commonly assessed grade which is normally done by Nottingham Modification of Scarff-Bloom-Richardson (SBR) grading system. The morphological features assessed in course

of grading are tubule formation, nuclear pleomorphism and calibrated mitotic count. Each feature is given value 1 (favorable) to 3 (unfavorable), and a combined score (total score of all three categorical features) is computed. Depending on the combined score value, tumor is graded as grade 1 (3-5), grade 2 (6-7) and grade 3 (8-9) (70). Table 3 summarizes the grading details.

Table 2: Anatomical Staging of Breast Cancer

Stages		Definition
Stage 0		Ductal Carcinoma In Situ
Stage I	IA	Primary invasive tumor with a size of $\leq$20 mm No nodal involvement
	IB	Nodal micrometastases ($>$0.2 mm, $<$2.0 mm) with or without $\leq$ 20 mm primary tumor
Stage II	IIA	Movable ipsilateral Level I, II lymph node metastases with $\leq$20 mm primary tumor; Or $>$ 20 mm, $\leq$ 50 mm tumor with no nodal involvement
	IIB	Movable ipsilateral Level I, II lymph node metastases with $>$20 mm, $\leq$ 50 mm tumor; Or $>$ 50 mm tumor with no nodal involvement
Stage III	IIIA	Movable ipsilateral Level I, II lymph node metastases with $>$50 mm tumor; Or any size primary tumor with fixed ipsilateral Level I, II or internal lymph node metastases
	IIIB	Primary tumor with chest wall and/or skin invasion
	IIIC	Any size primary tumor with supraclavicular or ipsilateral Level III lymph node metastases; Or with ipsilateral Level I, II and internal lymph node metastases
Stage IV		Any case with distant organ metastasis

Notes: 1). Lobular carcinoma in situ is now considered benign thus removed from the breast cancer staging system.
2). The Anatomic Stage Group is to be used when biomarker tests are not available.

Source: Feng Y *et al.* (56)

Table 3: Histologic Grading of Invasive Cancer by SBR, Nottingham Modification System

Histologic Grade, G	G Definition
GX	Grade cannot be assessed
G1	Low combined histologic grade (favorable), SBR score of 3-5 points
G2	Intermediate combined histologic grade (moderately favorable), SBR score of 6-7 points
G3	High combined histologic grade (unfavorable), SBR score of 8-9 points

1.5.1.4. Diagnostic Tests for Breast Cancer and Treatments

Diagnostic imaging is conventionally used approaches for breast cancer diagnosis. This includes mammography, ultra-sound, Magnetic resonance imaging (MRI) and Positron-emission tomography (PET). However, physical examination is also still used as a primary diagnostic particularly in resources-limited settings. Mammography is the most common used diagnostic tool for breast cancer especially for women after menopause. However, it is not suitable for younger women and women with dense breasts. Ultra sound is normally opted to diagnose women with dense breasts and it is advantageous in its ability to differentiate solid from cystic lesions (69).

MRI is usually used after mammography and Ultra sound fail to give clear results. It is also useful in diagnosing high risk people carrying BRCA1 or/and BRCA2 mutations. Further, MRI is important in assessing the response of tumor to neoadjuvant chemotherapy. PET is relatively more powerful as it can identify metastatic loci undetected by conventional methods. It can also assess the axillary nodal status essential in cancer staging prior to operation (69). After tumor is detected by imaging technology, histological examination of tumor biopsy normally ensues as a confirmatory test before further treatments.

The advances in genome-wide analysis and profiling of transcriptomes have given rise to development of multigene panels for diagnosis and prognosis of breast cancer. Currently available panels include Oncotype Dx 21-gene Recurrence Score (RS), Breast Cancer Index Score (BCI), the Genomic Grade Index (GGI), the Predictor Analysis of Microarray 50 (PAM50) Risk of Recurrence (ROR) Score and the Amsterdam 70-gene prognostic profile (Mammaprint) (56).

Screening for breast cancer is very useful in detecting the disease at the early and easily treatable stages. Breast self-examination and mammography has traditionally been used as a screening approach worldwide, and are still useful in Tanzanian settings. However, these tests suffer some limitations in terms of sensitivity and specificity especially in detecting disease at very early stages (71). Genetic testing for mutation in the specific panel of genes is more promising approach for screening and identifying the people at risk of developing breast cancer. Mutations in BRCA1 and BRCA2 genes are currently useful targets in genetic screening for breast cancer. The screening for mutations in some other genes such as TP53, CHEK2, PTEN, CDH1, STK11 and PALB2 also provides an important insight about the risk of breast cancer (56). However, the practicality of genetic testing in most African settings is far challenging due to their expensive costs which are unlikely to be covered by health insurance plans.

Hormonal therapy is a common therapeutic approach for breast cancers. In many cases, breast cancer expresses at least one of the three receptors; PR, ER and HER2. Prior to treatment, receptor typing is conducted to identify which receptor is highly expressed by cancer cells, and depending on the receptor type, appropriate hormone antagonists or targeted monoclonal antibodies is chosen. Tamoxifen is the commonly used estrogen antagonist for treating ER expressing tumors. Herceptin, a monoclonal based therapy targeting HER2 protein is normally useful for treating high HER expressing tumors (56). However, some breast cancers express neither receptors, and treatment options for these are restricted to conventional chemotherapy and radiotherapy. In Tanzania, most breast cancer patients present for clinical service at late stages, and mastectomy is the first common consideration for treatment followed by adjuvant therapy, which may include hormone therapy, chemotherapy or radiotherapy.

1.5.2. Carcinogenesis, Immunosurveillance, and Immunoediting

Carcinogenesis stems from deregulation in cell cycle control mechanism leading to uncontrolled cellular proliferation (72). Cell cycle entails a series of events driven by phosphorylation of important proteins by cyclin dependent kinases (Cdk), a group of phosphorylating enzymes active when they are in association with cyclin molecules (73). These cell cycle events are controlled at specific checkpoints which ensure the completeness of phase-specific events before

entering in the next phase (73). Cell control mechanism regulates spatial and temporal cell growth by balancing the activities of growth-promoting and growth-inhibiting proteins.

Growth promoting proteins are encoded by genes collectively referred to as proto-oncogenes, while the genes encoding proteins that inhibit the cell to grow are called tumor-suppressor genes. The regulation of expression and activities of these genes products is important for a cell to grow when the environments are conducive and to stop growing when the environments are not favorable. The cell aberrations leading to carcinogenesis upregulate and/or downregulate the activities of proto-oncogene and tumor-suppressor gene products respectively (74).

Aberration in a cell cycle control mechanism is not the sole driving force behind cancer development. The host immune system plays an important role in the pathogenesis of cancer. Many precancerous cells are kept in check and get destroyed by the immune system before they develop into cancer in immunocompetent host (75). Therefore, it is the interplay between cellular transformation and host immune response to these transformed cells which makes cancer development possible (76).

The implication of host immune system in development of cancer was firstly put forward by Burnet and his colleague in 1950's in what they called immune surveillance theory. According to the theory, the immune system recognizes the antigens on tumor cells and mounts the response against these altered cells, killing them before they grow into malignancy (77). There is a physiological logic behind this theory because, owing to high chance of carcinogenic mutations in large animals with long lifespan, there should be an evolutionary mechanism of getting the body rid of these mutation caused, altered and harmful cells (78).

There are many studies supporting the importance of host immune system in development of various cancers. Immunocompromised animals have higher risk of developing tumors compared to the immunocompetent counterparts (79). People with suppressed immune systems due to HIV infection have higher risk of developing oncogenic virus related tumors such as Cervical cancer, Hodgkin and non-Hodgkin lymphoma, Kaposi sarcoma and adenocarcinoma associated with HPV, EBV, HHV8 and HBV/HCV infections respectively, and even other tumors not related to viral infections (78).

Furthermore, people receiving immunosuppressive drugs meant to prevent organ rejection prior to transplantation have been reported to have higher risk of developing cancer (80,81). In murine model immunocompromised by knocking out RAG gene which is important in maturation of both B and T cells, the experimental group is highly susceptible to tumor development, both spontaneous and induced tumors (82). Studies show that immunocompetent mice exhibit high ability to reject the tumor transplanted in their bodies, compared to the immunocompromised counterpart (82).

It is now evident that in course of oncogenesis, tumor cells express some antigens capable of eliciting immune response. The tumor antigens can be divided into two groups. One group consists of antigens uniquely expressed on tumor cells and not on the other normal cells. These kinds of antigens are referred to as Tumor specific transplantation antigens (TSTA). TSTA arise from mutations preceding tumorigenesis, leading to abnormal intracellular proteins which can be processed into novel peptides and expressed on the tumor cell. The second group consists of antigens referred to as Tumor associated transplantation antigens (TATAs). These are molecules overexpressed in tumor cells as compared to normal cells. Also it includes those antigens which are only expressed by cells at a given developmental stages, but absent in the adult. The reactivated expression of such genes gives rise to proteins which may elicit immune response (83).

The immunogenic potential of these tumor antigens can be appreciated by tumor infiltration by immune cells. The analysis of most tumors reveals the presence of NK cells, T helper (Th), CTL and other regulatory immune cells in tumoral environments (84). Owing to their tumor rejection role in the host tissue, the amount of tumor infiltrating lymphocytes has been used as a prognostic indicator for cancer patients (85). In most cases, higher amount of tumor infiltrating lymphocytes, particularly Th, CTLs and NKp46+ cells, but not regulatory immune cells are associated with positive prognosis of cancer patients (86,87).

The immune system seems to have dual role in cancer pathogenesis. Not only does it act to reject tumor progression, but also is involved in the dynamic processes that ultimately favor tumor progression. This dynamic tumor-immune cells interaction which impacts the shift in nature of immune response to tumor cells and ultimately leading to tumor progression is referred to as

immunoediting (77). Immunoediting process is divided into three phases: Elimination, Equilibrium and Escape (78).

In elimination phase, the immune system mounted against tumor cells successfully keeps in check the growth of most tumor cells. In equilibrium phase, a dynamic balance between immune system mediated tumor cells eradication and the tumor cells variants not recognizable by immune cells exists. In escape phase, the colonies of tumor variants not recognizable by host immune system proliferate leading to clinical manifestation of malignant tumor (77,78). In course of immunoediting process, tumor microenvironment, some regulatory immune cells, and some molecules participate in suppressing the immune function leading to tumor development. Among other immunosuppressive molecules such as Forkhead box3 (Fox3), Programmed death ligand-1 (PD-L1) and Interleukin-10 (IL-10), HLA-G has been shown to have immunosuppressive functions which in turn favor tumor development in cancer patients (16,17,53,88).

1.5.3. The Biology of Human Leukocyte Antigen-G (HLA-G)

Human leukocyte antigens are cell surface proteins which were firstly discovered in 1930's by Peter Gorer as the antigens which take place in rejection of transplanted tissues (89). These antigens are encoded by genes clustered within an extended DNA stretch at chromosome number 6 in humans (90). Major histocompatibility complex (MHC) is the general name for these groups of genes, and HLA is exclusively used for human MHC. H-2 complex is a name for MHC complex in mice. HLA complex is divided into three regions, HLA-1, HLA-2 and HLA-3. HLA-1 and 2 encode protein responsible for presentation of antigenic peptides on the cell surface which elicit immune response. HLA-1 molecules are expressed by all nucleated cells and they present antigenic peptides derived from endogenous pathway, while HLA-2 molecules are exclusively expressed by APCs which present antigenic peptide derived from exogenous pathway (91). HLA-3 genes encode various secretory molecules essential in immune system function, such as components of complement system, heat shock proteins and some lymphokines (90).

The HLA class I molecules can be divided into classical and non-classical HLA class I. The antigenic presentation function pinpointed above is performed by classical HLA class I (92). Unlike classical HLA class I, non-classical class I HLA are restrictively expressed in some

specific tissues and display functions different from that of classical HLA class I molecules. Non classical HLA class I include HLA-G, HLA-E and HLA-F. HLA-G is said to have a suppressive effect on immune cells function, and this effect is important in protecting a developing semi-allogenic fetus against maternal immune attack (18). The immunosuppressive effect of HLA-G has also been observed in some pathological conditions associated with impairment or reduced immune functions such as cancer, viral infections and autoimmunity (23).

1.5.3.1. Gene Organization, Protein and Isoforms

The gene encoding HLA-G consists of seven introns and eight exons, but its primary transcript contains 7 exons. Exons 2, 3 and 4 correspond to extracellular domains α1, α2 and α3 respectively in the expressed protein. While exon 5 encodes for peptides that make up transmembrane region of HLA-G protein, exon 6 contains information for a short cytoplasmic tail composed of six amino acids. Exon 1 encodes peptides that act as a signal for directing the movement of HLA-G to the appropriate site. Exon 7 is not transcribed into primary transcript, and exon 8 makes 3'untranslated region of HLA-G mRNA. HLA-G protein encoded by a full transcript is 39kDa by molecular weight (23).

The post-transcriptional processing of HLA-G primary transcript can, by alternative splicing produce seven isoforms of HLA-G protein, HLA-G1, HLA-G2, HLA-G3, HLA-G4, HLA-G5, HLA-G6 and HLA-G7. Isoforms HLA-G1 to –G4 are membrane bound for they possess cytoplasmic and transmembrane domains, but HLA-G5 to –G7 lack transmembrane domains and hence exists in soluble forms (93). HLA-G1 has all three (α1, α2 and α3) extracellular domains, HLA-G2 has two extracellular domains for it lacks domain α2, HLA-G3 has one extracellular domain as it lacks domains α2 and α3, and HLA-G4 lacks domain α3 only and so it has two domains (23). HLA-G5, -G6 and G7 are soluble forms of membrane bound HLA-G1, -G2 and –G3 respectively as they possess respective extracellular domain (s), but lack transmembrane and cytoplasmic domains counterparts. Additionally, HLA-G5, –G6, and –G7 retain small translated parts of introns; 4, 4 and 2 respectively (23) which are exploited for immunoassay analysis of soluble forms of HLA-G (Figure 2).

Apart from being expressed from mRNA alternatively spliced from primary transcript, soluble forms of HLA-G can be derived from membrane-bound forms shed from their cell membranes (93). The shedding process may be catalyzed by proteolytic cleavage of HLA-G from the cell

membrane by metalloproteases (94,95). Some other soluble forms of HLA-G exist as secretory ones enclosed into vesicles (96). The vesicular form of soluble HLA-G is probably capable of fusing with plasma membranes of other cells and be taken up by such recipient cells, thus capable of shuttling HLA-G molecules between cells. A cell not expressing membrane-bound HLA-G can take up HLA-G expressed on the other cell through a process called trogocytosis (97).

To be able to present antigenic peptides, HLA class I molecules associate with β2-microglobulin, which act as a stabilizer. Only HLA-G1 and HLA-G5 possessing all three extracellular domains can bind β2M protein, and some researchers suggest that HLA-G1 is potentially capable of presenting antigenic peptides (98). Apart from those forms of HLA-G, the molecules can exist as dimers or even trimer. Dimerization is made possible due to existence of some cysteine residues in α1 and α2 domains which can form disulfide bond between two or more HLA-G molecules (99).

Tronik *et al*. reported the possibility of existence of some HLA-G isoforms other than the conventional seven isoforms known so far. By working with clear cell renal cell carcinoma, immunoassay and transcriptome analysis of HLA-G reveals the existence of HLA-G isoforms lacking all alpha domains but having extended 5' part (100). It is important to explore more about this possibility, as some novel HLA-G isoforms might be important for diagnosis or treatment of cancer or other pathological conditions.

HLA-G seems to be less polymorphic compared to classical HLA class I molecules. To date, only 47 HLA-G alleles are known and 15 distinct proteins (23). Some polymorphisms do not affect the sequence amino acids in the protein (conservative) while others significantly change the amino acid sequence hence modifying the structural and functional aspects of expressed proteins. The variation in amino acids content of HLA-G is observed in the extracellular alpha domains. Of note, the rare G*01:13N allele consists of stop codon in exon 2 resulting from substitution of Cytosine to Thymine at position 54. As a result, this allele does not produce any protein. The allele has been found in some African people (23) and studies on these people could shed much light on the role of HLA-G in cancer development and pregnancy associated complications such as eclampsia and recurrent spontaneous abortions.

Many polymorphisms exist in the regulatory sequences of HLA-G gene, and these have been investigated in relation to susceptibility of various diseases. The polymorphisms in the upstream part of the gene, particularly in the promoter region seem to affect the transcription of HLA-G gene. The polymorphisms existing in the 3'untranslated region have been suggested to affect post-transcriptional stability of HLA-G mRNA (101). 14bp insertion/deletion and +3142 G/C polymorphisms both located in 3'UTR are among the most studied polymorphisms in relation to various cancers development (102), autoimmune diseases (103), recurrent spontaneous abortion (104), preeclampsia (105), and sepsis (45).

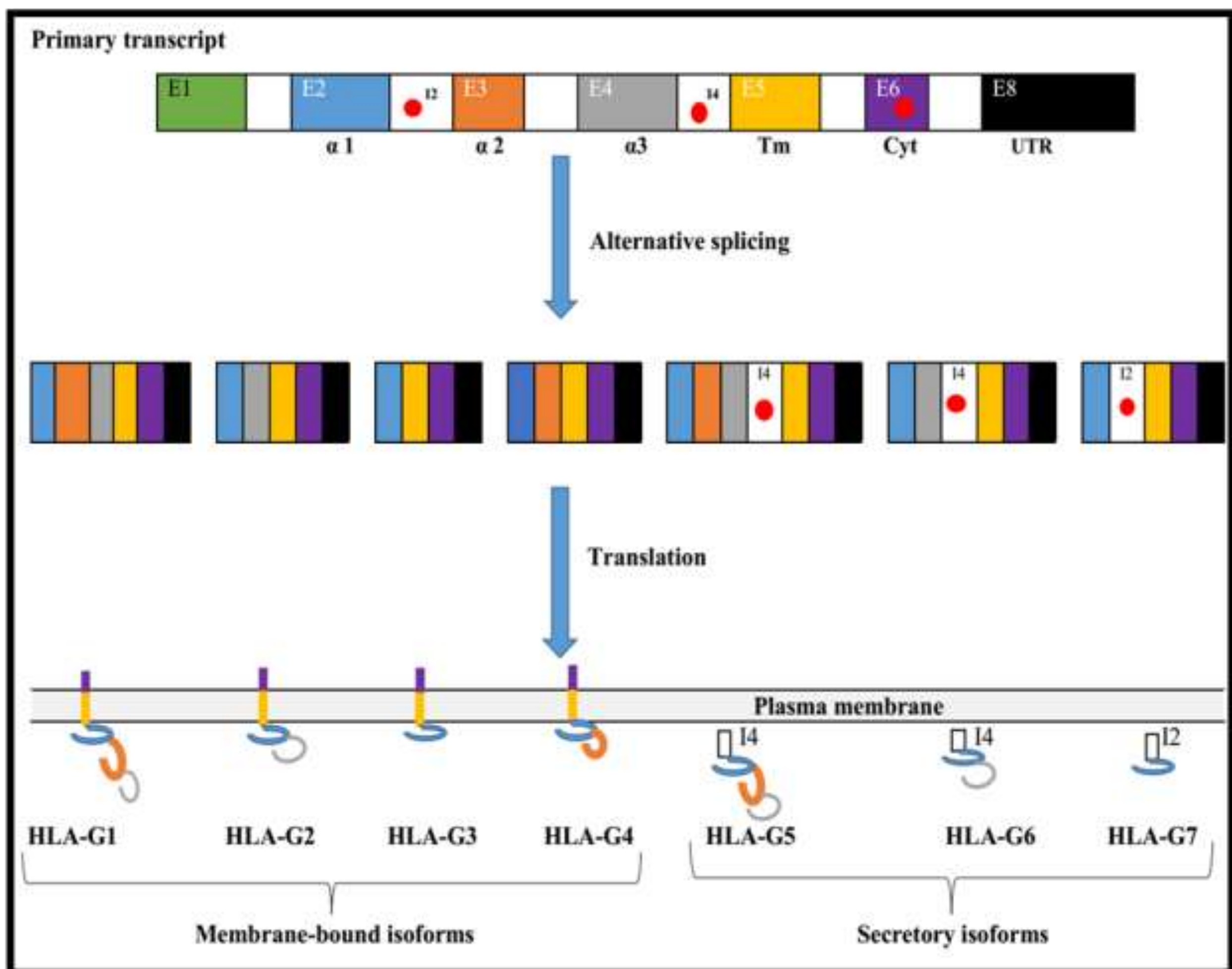


Figure 2**:** *HLA-G isoforms translated from alternatively spliced exons in HLA-G primary transcript. HLA-G1 to –G4 are membrane bound because they possess transmembrane region anchoring them to the membrane and cytoplasmic domain. HLA-G5 to –G7 are soluble ones as they lack transmembrane domain. Intron 2 intervening exons 2 and 3, and intron 4 intervening exons 4 and 5 contain stop codons (red dots) and are retained in mRNAs for HLA-G5, -G6 (intron 4) and –G7 (intron 2). Termination of*

translation at these stop codons makes some parts of introns to be incorporated into HLA-G5, -G6 and –G7 as short tails.

1.5.3.2. Regulation of HLA-G Expression

HLA-G expression is influenced by various transcriptional factors with cognate binding elements in the HLA-G gene regulatory sequences. The regulatory sequence in the upstream region of HLA-G gene is somehow different from that of classical HLA-G class I genes, thus responding to transcription factors differently. Unlike the classical HLA class I, upstream regulatory region (URR) of HLA-G bears modified form of enhancer A which cannot be induced by NK-kB (106). Also, the IFN stimulated response element (ISRE) present in classical HLA-G class I genes is deleted in HLA-G URR, and so cannot respond to IFN-gamma stimulation (107).

Apart from that, HLA-G URR contains three cAMP response elements (CRE), heat shock elements, progesterone response element and *ras* response element (RRE) (108). Ferreira *et al.* reported a distant regulatory sequence; enhancer L which specifically control transcription of HLA-G in trophoblasts. Deletion of this enhancer by CRISPR/Cas9 led to no expression of HLA-G (109).

Stress is considered to be an important environmental influence of HLA-G expression. In a low oxygen concentration (hypoxia), most cells respond by upregulating hypoxia inducible factor (HIF-1). HIF-1 binds to hypoxia response elements (HREs) present on target genes, inducing the transcription of proteins that enable the cell to survive in that abnormal environment (110). *In silico* analysis has revealed the existence of HREs in URR, 3' UTR, and even in exon 2 of HLA-G gene. Tumoral and trophoblast tissue microenvironments are characterized by low oxygen content (111), possibly contributing to upregulation of HLA-G by associated cells. The transcription factor, CRE binding protein-1 which binds to CRE is ubiquitously expressed in human cells, thus potentially inducing HLA-G transcription in most cells (112). Supported by some studies reporting the presence of HLA-G transcripts and not proteins in some normal cells (113,114), regulation of HLA-G at post-transcriptional level holds to be a prevalent one.

The most reported post-transcriptional regulation of HLA-G expression is mediated by miRNAs. These are short oligonucleotide RNAs which upon recognizing and binding to the

complementary sequence on mRNA, recruit ribonucleases which degrade the mRNA before its translation. In some in-vitro studies, overexpression of miRNA such as miR-148 and -152 in tumor cells or trophoblasts leads to significant downregulation of HLA-G molecule (115–118). Mori *et al*. reported that miR-365 from human trophoblast is overexpressed in hypoxic environment and downregulates expression of HLA-G (119). These findings contradict with the fact that hypoxia mediate upregulation of HLA-G through HIF-1 and hence elevating the level of HLA-G in trophoblast tissue (110).

HLA-G is also regulated epigenetically through DNA methylation and histone acetylation. DNA methylation in the promoter region results into downregulation of the respective genes, while histone acetylation brings about spatial decondensation of chromosome, leading to access of regulatory sequences in the decondensed region by respective transcription factors, and ultimately upregulating the associated genes. Holling *et al*. demonstrated that histone acetylation, and not DNA methylation is responsible for regulation of HLA-G expression by comparing the methylation and histone acetylation status between HLA-G expressing cell line (JEG) and HLA-G deficient cell line (JAR). The DNA methylation level was observed to be the same between the two cell lines, while histone acetylation was observed to be higher in HLA-G expressing JEG cell line (120).

The findings that DNA methylation is not involved in regulating HLA-G expression is supported by the work by Swets *et al*. (121). Although DNA methylation is reported not to have direct epigenetic regulation of HLA-G, some studies show that it indirectly does through regulation of miRNAs that affect the stability of HLA-G mRNA(117,122). Any mechanism that upregulates the expression of miRNAs which mediates HLA-G mRNA degradation is indirectly likely to regulate the expression of HLA-G.

1.5.3.3. Genetic Influence of HLA-G Polymorphism on its Expression

Some people are genetically predisposed to have higher expression of HLA-G compared to others. The genetic difference can affect the expression of HLA-G at both transcriptional and post-transcriptional levels. Most polymorphisms affecting the expression of HLA-G have been identified in regulatory regions, both 5'URR and 3'UTR. Polymorphisms in 5'URR affect the binding of transcription factors to their cognate regulatory sequences, while those in 3' UTR affect the stability of HLA-G mRNA after being transcribed (106). Owing to the implication of

upregulated HLA-G expression in different pathological conditions, these polymorphisms have been studied as the genetic determinants of the respective pathological conditions such as different cancers (123), autoimmune diseases (124), sepsis (45), and recurrent spontaneous abortions (125).

Promoter region of HLA-G gene contains at least 29 SNPs which may affect its transcriptional level by affecting the binding of transcriptional factors to regulatory elements present in the promoter. Examples of such SNPs include -666G/T, -689A/G, -716T/G, -725C/G/T, -762C/T, -810C/T, -964G/A, -990G/A, -1140A/T, -1179A/G and -1306G/A (107). -725C/G/T SNP is among the most commonly studied SNPs, and -725G allele variant has been associated with higher level of transcription of HLA-G gene than the rest two (125). However, SNPs -725C/G/T and -716T/C have been studied in relation to non-small cell lung cancer and no association of both two have been found (126).

Some notable polymorphisms in 3'UTR include 14bp Insert/Delete, 3142G/C, +3010C/G, 3027A/C, 3035C/T, +3187A/G and +3196C/G. 14bp Insert/Delete polymorphism is one of the common studied polymorphism which is considered to affect the stability of HLA-G mRNA. Deleted form is considered to produce a more stable HLA-G mRNA compared to Inserted one (102). The impact of allelic forms and genotypes of this polymorphism is controversial in pathological conditions thought to be mediated by elevation of HLA-G expression, with some researchers reporting associations (36,42) while others reporting no associations (102,103). The meta-analysis conducted by Li *et al*. showed that, with pooled analysis, 14bp Ins/Del polymorphism is not associated with cancer development. However, in sub-group analysis, 14Ins/Del seems to be associated with breast cancer (44).

The other common polymorphism most studied is +3142G/C polymorphism. It is important in post-transcriptional regulation of HLA-G expression. It affects the stability of HLA-G mRNA through modulating its affinity to miRNAs. The miRNAs mediating HLA-G mRNA degradation; miR-148a, miR148b and miR-152 are said to have more affinity to G allele compared to C allele (127). Because of this difference in affinity, mRNA bearing C allele is more likely to make its way to translation than mRNA bearing G allele.

The possible influence of this polymorphism on development of pathological conditions associated with upregulation of HLA-G expression is also somehow contradictory. Zidi*et al*.

(127) reported +3142G allele to be a risk factor for breast cancer development, but its corresponding genotype +3142G/G to be a protective one. +3142G allele has also been associated with prostate cancer (128) and sepsis (45) while +3142C is considered to be protective against Rheumatoid arthritis (129). Some other studies report no association between +3142G/C polymorphism and HLA-G elevation associated pathological conditions (47). Furthermore, the in vitro study by Manaster *et al*. reported that +3142G/C polymorphism does not affect HLA-G mRNA targeting by miRNAs (115).

The +3142G/C SNP is among the most frequent variation sites in HLA-G 3'UTR, and has been found to be in a strong linkage disequilibrium (LD) ($r^2 \geq 91$) with +3010C/G in both African, Europe, Asian and American populations (130). The frequency distributions of +3142G/C alleles and genotypes in different populations have been explored from 1000 Genomes and HapMap projects, and are presented in Figures 3 and 4. No information regarding distribution of +3142G/C variants in any Tanzanian population is currently available.

The influence of 3'UTR haplotypes of HLA-G gene on HLA-G expression; such as UTR-1, UTR-2, UTR-3, UTR-4, UTR-5, UTR-6, UTR-7, UTR-8 and UTR-18 has been studied by Poras *et al*. They reported the differential response to transcription activating intracellular factors by HLA-G expressing and non-HLA-G expressing cell lines. They showed that UTR-5 and UTR-7 haplotypes are associated with higher expression of HLA-G, UTR-2, -3, 4, 18 haplotypes have intermediate impact on HLA-G expression and UTR-1 haplotype's impact on HLA-G is very low (43).

The genetic polymorphisms residing in coding region of HLA-G have also been investigated whether they influence expression of HLA-G. Khorammi *et al*. reported that G*01:01:03:01 and G*01:01:08 have a protective effect against adenocarcinoma (131). Amor *et al*. found that, when in heterozygous form, HLA-G*010101 and G*010401 are associated with non-small cell lung cancer (132).

Therefore, given such heterogeneity and contradictions among the findings on the influence of HLA-G polymorphism in the development of HLA-G associated pathological conditions, further studies are needed to give a big picture on whether these polymorphisms truly influence the outcome of such conditions. Furthermore, most of studies about HLA-G polymorphisms in relation to pathological conditions such as cancer have been done mostly in Brazil, and some in

Europe, China and Korea. Some few studies have been done in Tunisia, but most African ethnic groups have not been included in such studies. Increasing the number of such studies coupled with use of different ethnic groups will deepen our understanding on the potential influence of these polymorphisms, particularly HLA-G +3142G/C on cancer development.

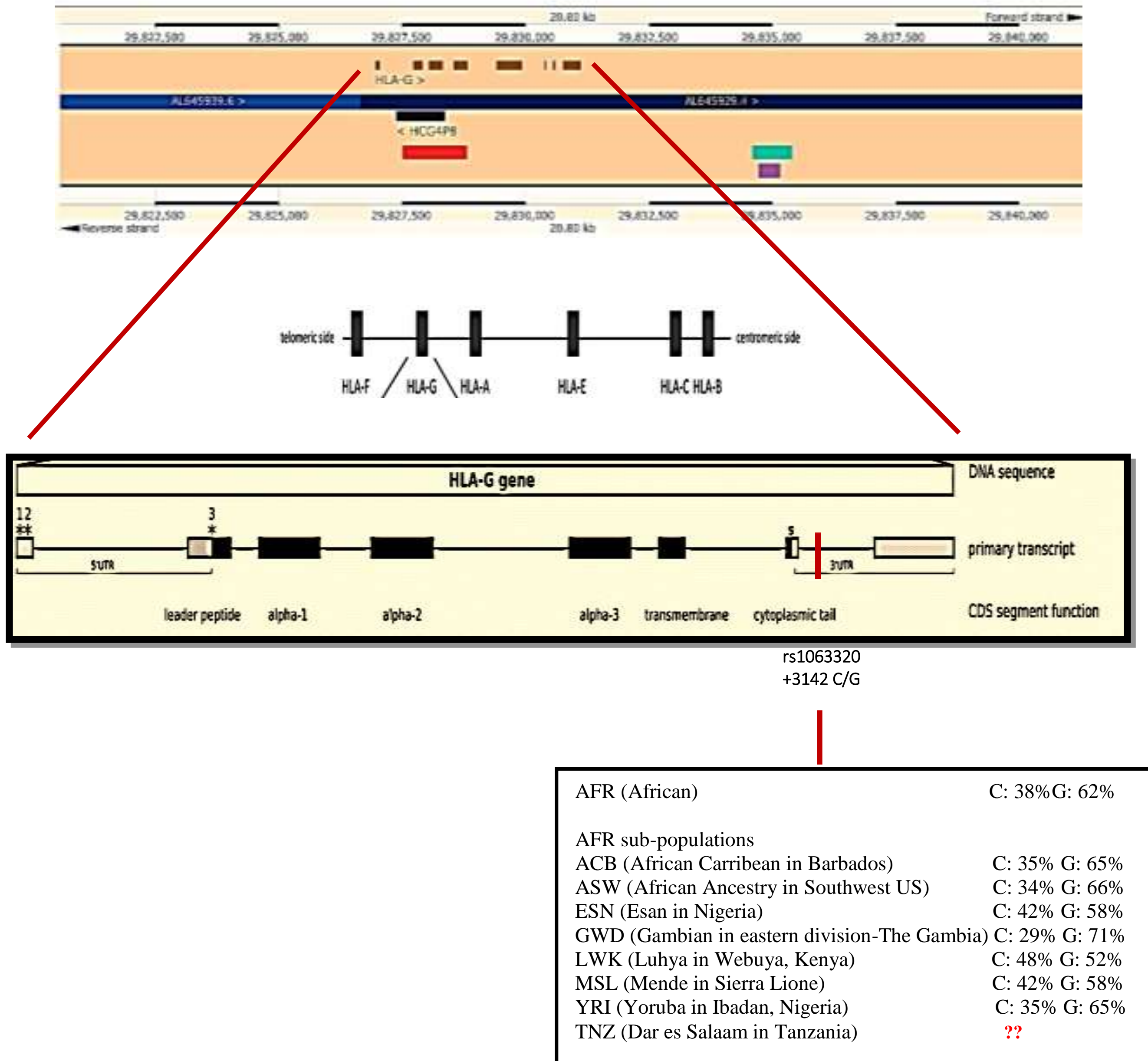
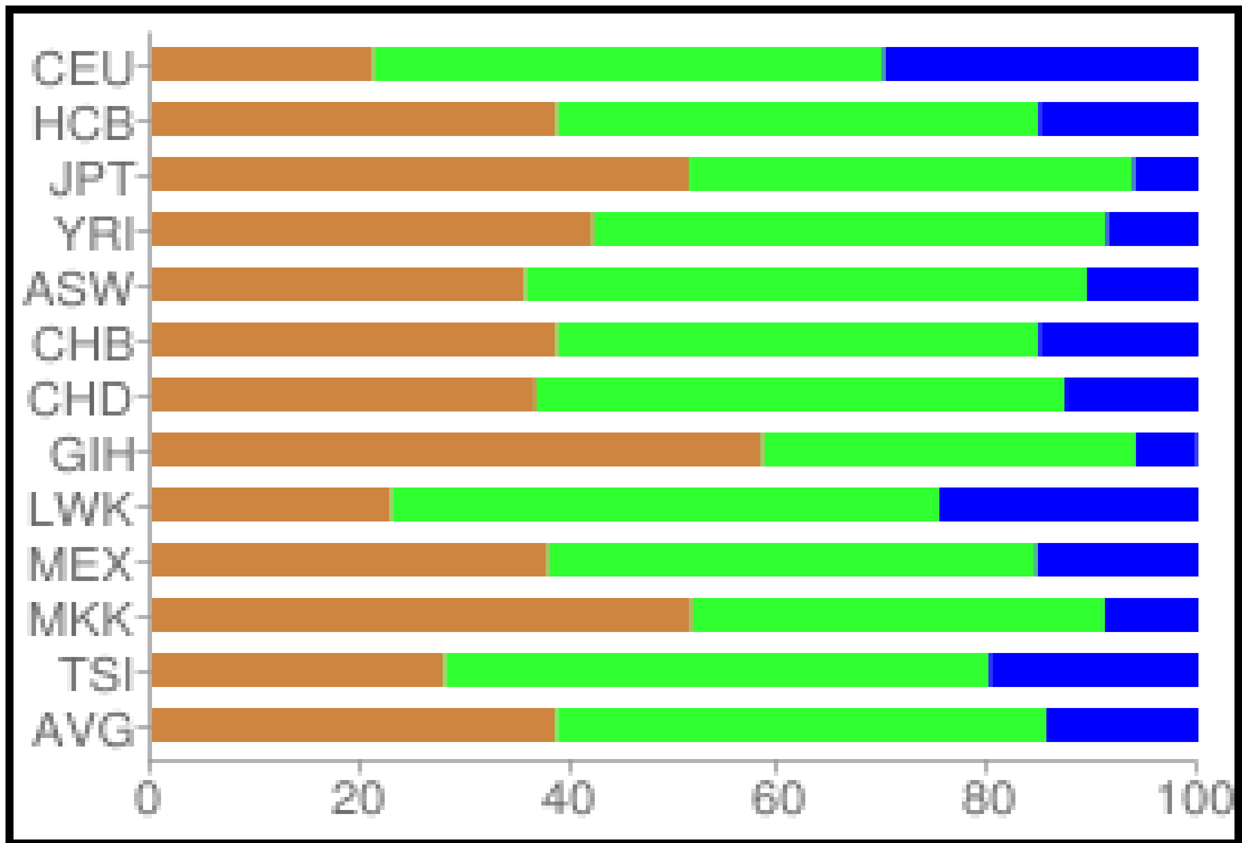


Figure 3: *1000 Genomes Project Phase 3 allele frequencies for 1063320. 1000 Genomes Project Phase 3 allele frequencies for Africa in general and African Subpopulations are presented. Data was taken from https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/.*



(C:C) (C:G) (G:G)

Figure 4: *Population diversity box representing populations studied in the International HapMap project for rs1063320. (https://www.snpedia.com/index.php/Rs1063320).*

Key**:**

- CEU - European -Utah residents with Northern and Western European ancestry
- CHB - Han Chinese
- JPT - Japanese in Tokyo, Japan
- YRI - Yoruba in Ibadan, Nigeria (Africa)
- ASW - African ancestry in Southwest USA
- CHD - Chinese in Metropolitan Denver, Colorado
- GIH - Gujarati Indians in Houston, Texas

- LWK –African Luhya in Webuye, Kenya (Africa)
- MEX - Mexican ancestry in Los Angeles, California
- MKK - Maasai in Kinyawa, Kenya (Africa)
- TSI - Toscani in Italia
- AVG-Mathematical average of all samples from above groups

Table4: Association between selected HLA-G polymorphisms and some Pathological conditions

HLA-G Polymorphism	Reference sequence	Condition	Allele/Genotype	Association	Reference
+3142C/G	rs1063320	Breast cancer	+3142G/G	Negative (Protective)	(127)
+3142C/G	rs1063320	Colorectal cancer		No	(47)
+3142C/G	rs1063320	Sepsis	+3142G	Positive	(45)
+3142C/G	rs1063320	Prostate cancer	+3142G	Positive	(46)
+3142C/G	rs1063320	Rheumatoid arthritis	+3142C	Negative (Protective)	(103)
14InDel	rs371194629	Pre-ecamp/ecamplisia		No	(105)
14InDel	rs371194629	Sepsis	14bp Ins	Positive	(45)
14InDel	rs371194629	Rheumatoid arthritis		No	(103)
14InDel	rs371194629	Prostate cancer		No	(46)
14InDel	rs371194629	Breast cancer		No	(102)
14InDel	rs371194629	B cell lymphoma	14bp Ins	Positive	(123)
+3196C/G	rs1610696	Colorectal carcinoma	+3196G	Positive	(47)
-725C/G/T		NSC lung cancer		No	(133)
-716T/G		NSC lung cancer		No	(133)
-725G/C		B cell lymphoma	-725G	Negative (Protective)	(123)

1.5.4. Immunomodulatory Functions of HLA-G Molecule

In most studies conducted under both physiological and pathological conditions, HLA-G seems to function by inhibiting normal immune cells functions. This function is responsible for normal physiological immune tolerance or viral/altered cells immune escape in pathological conditions.

The immunosuppressive function of HLA-G was firstly studied in search of its role in pregnancy maintenance as its expression was observed to be upregulated in trophoblasts. Nathalie *et al.* utilized monoclonal antibody specific to HLA-G on trophoblast cells co-cultured with semi-allogeneic NK cells. NK cells in anti-HLA-G treated cell culture showed higher cytotoxic activity compared to those in culture not treated with anti-HLA-G antibody (18). Anti-HLA-G neutralized the activity of HLA-G, allowing NK cell cytotoxicity to take place.

As the fetus carries some paternal derived molecules which can be recognized by maternal immune system as antigens, HLA-G induces maternal immunotolerance to this semi-allogeneic creature sparing it from immune cells mediated destruction (23). Many studies clearly demonstrate this fetus protective role of HLA-G, as downregulation of HLA-G in trophoblasts during pregnancy is associated with miscarriage. The recurrence miscarriages in many women are associated with the genetic configuration of HLA-G genotypes expressing low amount or no HLA-G molecules (104,125).

Further immunosuppressive role of HLA-G has been extended to autoimmune diseases and viral infections pathogenesis. In autoimmune diseases, the immunotolerance to the self-molecules is disrupted and the self-molecules are recognized and destructed by immune cells. Downregulation of HLA-G expression in Rheumatoid arthritis patients has been observed by Verbruggen *et al.* (134). Viral infected cells normally present the viral derived antigens on their surfaces eliciting the immune response which subsequently kills virus bearing cells. However, many viruses exploit different mechanisms to escape from immune recognition, and upregulation of HLA-G have been observed in patients infected with HIV (135), and human cytomegalovirus (136).

The immunosuppressive role of HLA-G has been extensively studied in cancer development owing to its increased level in tumor tissues and patients' circulatory system. Both in vitro and in vivo studies have demonstrated that HLA-G on tumor cells inhibits immune response to them. Paul *et al*. (137) demonstrated the protective role of HLA-G on IGR melanoma cell lines against NK cell cytotoxicity. NK cells co-cultured with melanoma cell lines expressing upregulated level of HLA-G exhibited poor cytotoxicity against these allogeneic cells.

The similar effect is demonstrated by Loumagne *et al*. (17). In this in vivo study, the immunosuppressive role of soluble HLA-G was investigated by transfecting some tumor cell lines with immunogenic *hβ2m* molecule only and others with *hβ2m* and HLA-G5. After injecting

the transfected tumor cells in the immunocompetent mouse, tumor cells transfected with *hβ2m* only were rejected by the mouse immune system. However, the tumor cells transfected with both *hβ2m* and HLA-G5 could not be rejected by the mouse, indicating the protective role of HLA-G5 to tumor cells against host immune attack. HLA-G mRNA interference in tumor cells by shRNA also improves their susceptibility to NK cells cytotoxicity (138). Although many studies have demonstrated that HLA-G suppresses the function of NK cells, the recent study done by Veluchamy *et al*. reported that HLA-G and HLA-E do not affect the cytolytic ability of NK cells on cervical cancer cells (139).

However, the study on Qa-2, a molecule which is considered to be a murine homolog of HLA-G showed the opposite effect on tumor fate both in vivo and in vitro. Istéfani *et al.* (140) demonstrated this by injecting mouse with 4T1 breast cancer cells in syngeneic mouse and collecting developed tumors after 10, 17 and 24 days; and establishing tumor cell lines from the collected tumors. The expression of Qa-2 was reduced as tumor grew in vivo, and tumor derived cell lines showed also a reduced expression of murine HLA-G homolog, Qa-2. Supported by some other findings (141), this study raises question about the homology nature between Qa-2 and HLA-G.

HLA-G can exert its immunosuppressive effect directly or indirectly. Direct exertion is achieved by interaction of HLA-G molecule with specific receptors on immune cells which transmits inhibitory signal (15). The notable inhibitory receptors for HLA-G are ILT4, ILT2and KIR2DL4 which are widely expressed by different immune cells. Cells expressing ILT2 include B cells, T cells, NK cells, monocytes and dendritic cells. Dendritic cells, monocytes and macrophages express ILT4 while KIR2DL4 is expressed by some T cells and NK cells (23). These receptors transmit the inhibitory signal through Immunoreceptor tyrosine based inhibitory motif (ITIM). Downregulation of ILT2 on NK cells rescues their cytotoxic effect against tumor cells (11).

The binding of HLA-G to most receptors is through α3 domain which is present in some membrane- bound and soluble forms of HLA-G, implying the systemic effect of soluble HLA-G on immune system impairment. Interaction of HLA-G with ILT2 on B cells inhibits both naïve and memory B cells' proliferation, differentiation and secretion of Immunoglobulin by arresting the cell at G0/G1 of the cell cycle (16). Soluble forms of HLA class I molecules induce apoptosis

of CD8+ T and NK cells by interacting with CD8 molecule and upregulating the expression of surface and soluble FasL (142).

The indirect way by which HLA-G suppresses immune cells function is through activation of immune cellular differentiation to regulatory immune cells such as regulatory T cells, regulatory B cells, Myeloid derived suppressor cells (MDSCs) and DC-10 (143,144). All these regulatory cells function by suppressing the normal activities of immune cells. Furthermore, HLA-G indirectly impair the immune cells function through the direct uptake of HLA-G molecules from membrane of mHLA-G expressing cell by immune cells, a process called trogocytosis (97), which can subsequently induce cellular anergy, affect the cytokine production profile and expression of receptors. However, Ostapchuk *et al*. demonstrated that NK cells expressing HLA-G negatively modulate the cytotoxicity of other NK cells not expressing HLA-G through direct cell-cell contact, and not through altered cytokines produced by HLA-G bearing cells (145).

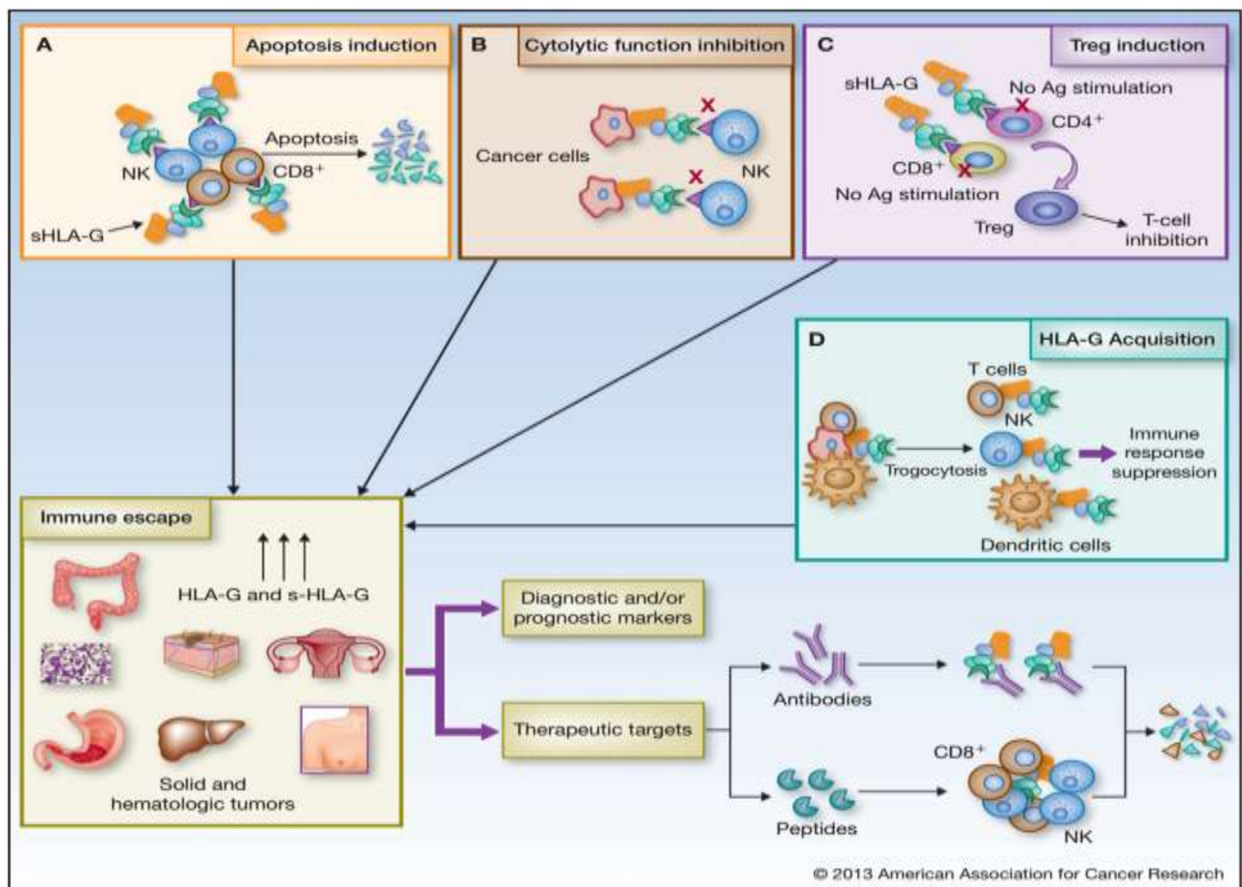


Figure 5: *Ways by which HLA-G induces immunosuppression(Source: Curigliano et al., 2013) (48). A: sHLA-G induces apoptosis when interacts with CD8 and NK receptors, B: HLA-G inhibits cytolytic*

ability of NK cell upon interacting with its inhibitory receptors; C: Interaction of HLA-G with CD8 and CD4 cells can transform them into regulatory T cells (Tregs); D: A cell devoid of HLA-G can acquire HLA-G from the surface of HLA-G-bearing cell, suppressing the immune function of the recipient (trogocytosis). All these ways make immune escape by solid and hematological tumors possible. This makes HLA-G a potential therapeutic target and biomarker for cancer diagnosis/prognosis.

1.5.5. Expression of HLA-G in Malignancy

Significant number of studies has been conducted to determine the expression level of HLA-G in breast cancer, bladder cancer, ovarian cancer, hematological malignancies and other types of cancers. In many cases, increased expression of HLA-G has been observed in tumor lesions as membrane-bound HLA-G (27,37,55,146,147) and in body fluids such as blood and ascites (33,36) as soluble HLA-G. HLA-G expression in cancer patients has been reported to be upregulated in both tumor cells and regulatory immune cells such as peripheral blood monocytes (54), some NK cells (145) and DC-10 (88). The elevated expression of HLA-G is further increased by some cancer treatments undertaken by patients such as Cytokine IFN-alpha (148) and DNA methyl transferase (DNMT) inhibitors (8).

The most common techniques that have been used in studying expression of HLA-G in relation to cancer are immunohistochemistry, ELISA and Flow cytometry. Immunohistochemistry is useful in studying the proportions of tumor cells expressing mHLA-G, ELISA for studying the level of soluble HLA-G in serum while flow cytometry is important when it comes to immunophenotyping of peripheral blood mononuclear cells (PBMCs). In most tumors, unlike classical HLA class I molecules, HLA-G is not evenly expressed throughout tumor tissue (55). Furthermore, the expression profile of HLA-G is different in different tumors and even in different regions of the same tumor (149).

Hypoxic environment in the tumor tissues is considered to be a factor behind upregulation of HLA-G expression. Hypoxic environment induces expression of HIF-1, the transcription factor which binds to their cognate hypoxic binding elements present in different genes whose expression is upregulated under low oxygen environment. HLA-G gene bears such binding elements in exon 2 and non-coding regions of HLA-G (150). The binding of HIFs to their cognate sequence elements upregulates the expression of respective genes. However, provided that hypoxic environment is present in solid tumors, upregulation of HLA-G expression in non-

solid cancers such as leukemia implies that some other mechanisms associated with oncogenesis are employed in upregulating the expression of HLA-G, possibly through altered cytokine production profile associated with transformation (151).

Many studies have investigated the relationship between expression level of HLA-G and some clinicopathological parameters of different cancer types. In most studies, HLA-G expression is independent to gender or age (35,37,88,152). Although it is well known that HLA-G expression is upregulated in pregnant women, the study by Rebmann *et al*. showed that the level of soluble HLA-G in breast cancer, malignant melanoma, ovarian cancer and glioma patients is independent to pregnancy status (54). Owing to its immunosuppressive role which is in favor of tumor development, many studies have investigated the association between degree of HLA-G expression and progression of tumors. Comparison between expression level of HLA-G in pre-cancerous cells and cancerous counterparts shows the increased level of expression of HLA-G in cancerous cells (29,107,153), indicating the influence of HLA-G in progression of tumor cells from precancerous state to cancerous state. However, the study conducted by Swets *et al.* (147) reported lower expression of HLA-G in tumor lesions of liver metastases compared to their colorectal primary lesions counterparts.

Some other studies however could not confirm the increased expression of HLA-G in tumors. Davies *et al.* investigated the expression of HLA-G in brain, breast, colon, melanoma and lung tumors, and could not detect HLA-G protein by both immunohistological and flow cytometry method. The study could not find the HLA-G expression on the leukocytes infiltrating these tumors as well (154). Similar results were found by Pangault *et al.* in which total 33 tumor tissues from breast, lung, liver, kidney, ovary and larynx cancers were characterized (155).

Real *et al.* studied the panel of tumors consisting 50 tumor tissues of different varieties and 31 tumor cell lines. Although mRNA transcripts for different isoforms of HLA-G were detected in most samples, no evidence of HLA-G protein was found in all tumor tissues and cell lines (156). In line with those findings, Frumento *et al.* could not find HLA-G protein expression in both melanoma and melanoma cell lines (157). Furthermore, Gao *et al*. reported that HLA-G protein is not or lowly expressed by myeloma cells, and this molecule does not mediate myeloma resistance to NK cell, but classical HLA-1 molecules do (158).

Furthermore, the study conducted by Amiot *et al*. compared the expression of HLA-G between normal and malignant hematopoietic cells. Although transcriptional activity of HLA-G gene was observed, HLA-G protein could not be detected neither on the cell surface nor in the cytosol (113). More and larger studies in more ethnic groups are required to solve this existing controversy regarding HLA-G expression in malignancy.

1.5.6. Soluble HLA-G Levels in Malignancy

The limited availability of reliable plasma derived biomarkers for different cancer types has been a reason behind many researchers' interests to investigate the level of soluble HLA-G (sHLA-G) in various cancer patients. Biomarkers found in body fluids such as blood and ascites are more suitable for diagnosis and prognosis as they are less invasive and risky.

The level of sHLA-G has been studied in different cancers. Chen *et al*. investigated the plasma level of sHLA-G in ductal breast cancer patients and found the drastically increased level of sHLA-G in patients compared to normal controls (37). Some studies show that increased sHLA-G expression is associated with triple negative status of breast tumors (127) while others report no such association (35). Mohammad *et al.* (159) showed the progressively increased level of soluble HLA-G from normal men, men with prostate benign tumor to prostate cancer patients.

The expression of sHLA-G in the ascites from breast and ovarian cancer patients was investigated by Singer *et al*. and the findings indicated significantly high level of sHLA-G in malignant ascites than that in benign ascites (160). Similarly, in the study conducted by Sun *et al*., malignant ascites obtained from liver cancer, lung cancer, colon cancer, renal cancer, ovarian, breast invasive ductal carcinoma, ampullary carcinoma and Hodgkin lymphoma patients contained significantly higher level of sHLA-G compared to that found in benign tumors obtained from liver cirrhosis and tuberculous peritonitis patients (33).

The plasmatic sHLA-G levels in lymphoproliferative disorders were investigated by Sebti *et al*. They found significantly increased levels of sHLA-G in 70% of B chronic lymphocytic leukemia, 45% of non-Hodgkin T lymphoma and 53% of non-Hodgkin B lymphoma (161). Non-significant increased level of sHLA-G in acute lymphoblastic leukemia pediatric patients, and its significant increased level following chemotherapy was reported by Motawi *et al.* (162). However, the study conducted by Gan *et al*. found the relatively similar levels of sHLA-G between bladder transitional carcinoma patients and normal controls (163).

While most studies show that HLA-G expression in the tumor tissues increases with an increase in tumor stage (28,31,37), studies on soluble forms of HLA-G in the serum show conflicting results. In some studies, elevation of soluble HLA-G in cancer patients' serum seems to be associated with tumor stage (35) while others report no such association (36,37). This difference might be attributed to confounding effect of HLA-G produced by peripheral blood monocytes, and the possibility of subjects in some studies being under treatments that upregulates expression of soluble HLA-G by different cells in peripheral blood.

1.5.7. HLA-G as a Diagnostic and Prognostic Biomarker and Potential Therapeutic Target

The fact that HLA-G expression is upregulated in the cancer patients has been utilized to seek for its diagnostic potential. In most studies conducted, sHLA-G is found to be significantly higher in the malignant tumor patients' plasma compared to that in normal healthy individuals (36,88,148,159). This difference in level of sHLA-G has been investigated whether it can be truly useful to discriminate tumor patients from normal people, or benign tumors from malignant ones. Selected findings about such discriminatory utility of sHLA-G are summarized in the Table 2.

A good diagnostic tool should be able to detect disease when truly present in patients, and detect no disease when truly absent in normal healthy individuals. That is to say, a diagnostic tool should have high, both sensitivity and specificity. The overall diagnostic performance, sensitivity and specificity for continuous variables like sHLA-G can be obtained through constructing the Receiver Operating Characteristic (ROC) curve. This is a curve of sensitivity against (1-specificity), and its area under the curve (AU-ROC) determines the overall diagnostic performance of the variable. The maximum AU-ROC curve is 1, meaning that both sensitivity and specificity are 100%, but this is not practical for the most diagnostic tools. The more the ROC-AU is closer to 1, the better is the diagnostic performance (164,165).

As it might be observed in the randomly selected studies presented in Table 2, most studies investigating the diagnostic potential of sHLA-G in different types of cancer report its good diagnostic performance, most reporting AU-ROC greater than 0.7. Some other studies even compare the diagnostic capability of sHLA-G with other common used tumor markers. sHLA-G is reported to significantly outperform CA and CEA as tumor markers for breast (35), gastric,

(36), colorectal, esophageal and lung cancer (34). Heidari *et al*. compared the diagnostic significance of sHLA-G against conventional PSA marker for prostate cancer diagnosis. sHLA-G is shown to have a better performance than PSA, and the authors recommend sHLA-G to be used as a supplementary to PSA in prostate cancer diagnosis (159).

Regarding the use of sHLA-G AU-ROC to infer tumor stage or grade, only few studies have investigated this. Many studies have been focusing on statistical association between sHLA-G in plasma and severity of tumor, and most of them report that sHLA-G is not associated with tumor stage/grade (33,36) although it is significantly elevated in tumor patients compared to normal healthy individuals. However, Sun *et al*. demonstrated that ascitic sHLA-G can be used to discriminate between benign and malignant tumors among primary liver, lung, colon, ampullary, renal, cervical, pancreatic, ovarian cancer and non-Hodgkin lymphoma as malignant tumors, and liver cirrhosis and tuberculosis peritonitis as benign tumors (33). Also, Jeong *et al*. showed the diagnostic performance of sHLA-G to differentiate metastasized cancer from other stages (35).

Apart from diagnostic utility, many studies have investigated the potential use of sHLA-G as a prognostic marker. Many studies have associated sHLA-G level with poor prognosis. Kirana *et al*. reported that higher sHLA-G level was associated with poor prognosis in colorectal cancer patients (166). Contrary results were reported in Rutten *et al*. study in which increased sHLA-level was associated with prolonged survival among high grade ovarian carcinoma patients (167). Some other studies have shown that sHLA-G can be helpful in monitoring the response to treatment by cancer patients. The study conducted by Sayed *et al*. reported a significant decrease in sHLA-G level among mastectomized breast cancer patients undertaking adjuvant therapy after 12 months of follow up. The few patients whose sHLA-G levels were still high were found to have had their cancers metastasized (38). Similarly, Rutten *et al*. found a significant decline in sHLA-G level following chemotherapy treatment of high grade ovarian cancer. The sHLA-G level increased to almost the initial level following recurrence (167).

König *et al*. discouraged the use of total sHLA-G as a prognostic marker, and emphasized the use of the subcomponents thereof (free and extracellular derived sHLA-G). The suggestions were based on their findings that free sHLA-G and extracellular derived sHLA-G had different prognostic values. While extracellular derived sHLA-G was found to be associated with breast cancer progression, free sHLA-G was associated with positive prognosis (96).

In B cell neoplasms, HLA-G expression is normally associated with better prognosis. This association has been attributed to the inhibitory effect of HLA-G exerted to the malignant B cells upon interacting with ILT2 receptor which leads to reduced proliferation ability of B cells, and hence improving the clinical condition (168). However, the findings from the study by Yong *et al*. in non-Hodgkin lymphoma patients showed that sHLA-G is not a good prognostic marker in clinical settings (169).

Due to abnormal rise of HLA-G expression in tumor tissues, many researchers are recommending to make this molecule “a target for immunotherapy”. In course of tumorigenesis, tumor cells downregulate the expression of classical HLA class I making them unable to present tumor antigen peptide on their surfaces to elicit T cells immune response (170). This downregulated expression of classical HLA class I make these cells lack some self-molecules, hence becoming susceptible to NK cytolytic attack. The upregulation of immunosuppressive molecule HLA-G by tumor cells circumvents this recognition and lysis by NK cells, making tumor progression possible (137). Therefore, an approach capable of downregulating expression of HLA-G by tumor cells could improve the efficacy of immunotherapy (117,118).

The approaches to intervene the interaction between HLA-G molecule on tumor cells and the respective receptors on immune cells have demonstrated an improved immune cells response to tumor cells. Blocking tumor HLA-G with monoclonal antibodies increased tumor lysis in mice injected with M8-HLA-G1 cell lines (171). Also, suppression of formation of regulatory T cells (CD4+/CD25high/Foxp3+ Tregs) and subsequent proliferation of allogeneic T cells were observed upon neutralizing sHLA-G molecule (172). Downregulation of HLA-G mediated by siRNA improved cytolytic ability of NK cells against human hepatocellular carcinoma cell line (173).

Ishibashi *et al.* investigated the possibility of priming HLA class II restricted immune response to tumor cells by using the peptide derived from HLA-G. The results are impressive because upon stimulating CD4+ HTLs with HLA-G derived peptide, they were able to recognize tumor cells bearing HLA-G and mediate immune response to tumor cells. The response is even elevated when HLA-G expression by tumor cells is further upregulated by treating tumor cells with DNMT inhibitor (5-AZA, 5-aza-20-deoxycytidine). Importantly, this study shows that

CD4+ HTLs possess cytolytic ability as they seemed to lyse tumor cells bearing HLA-G molecule (8).

In many studies, sHLA-G seems to be a promising biomarker in different types of cancer. The supplementary usage of this molecule to other conventional biomarkers for respective type of cancer could potentially improve the diagnostic outcome. However, the body of knowledge is lacking sufficient findings of that kind from African ethnic groups.

Table 5: Diagnostic performance of sHLA-G in some Selected Cancer Types

Condition	Discriminatory Utility	Sample	AU-ROC	COV	S_N (%)	S_P (%)	Population	Ref
Breast cancer	Patients Vs Healthy	plasma	0.89	19.4U/ml	92.5	70	Korea	(35)
NSCLC	Patients Vs Healthy	plasma	0.82	24.9U/ml	52.8	100	Caucasian	(132)
Gastric cancer	Patients Vs Healthy	plasma	0.730	84U/ml	27.2	95.4	China	(36)
Gastric cancer	Patients Vs Healthy	plasma	0.70	-	-	-	China	(88)
Carcinomas	Malignant Vs Benign	ascites	0.957	19.6µg/L	87.5	100	China	(33)
Breast cancer	Metastasis Vs Other stages	plasma	0.79	147.7U/ml	88.9	69.0	Korea	(35)
Colorectal cancer	Early stages patients Vs Healthy	plasma	0.97	49U/ml	94	100	China	(34)
Gastric cancer	Early stages patients Vs Healthy	plasma	0.91	49U/ml	85	100	China	(34)
Esophageal cancer	Early stages patients Vs Healthy	plasma	0.98	49U/ml	91	100	China	(34)
NSCLC	Early stage patients Vs Healthy	plasma	0.8	49U/ml	51	100	China	(34)
Breast cancer	Patients Vs Healthy	plasma	0.935	68.82U/ml	65.9	100	China	(37)
Breast cancer	Patients Vs Healthy	plasma	0.95	0.54 µg/L	88.1	100	China	(30)

AU-ROC: Area under receiver operating curve; COV: Cut-off value; S_N: Sensitivity; S_P: Specificity; NSCLS: Non-small cell lung cancer; Ref: Reference

2.0. MATERIALS AND METHODS

2.1. Type of Study and Study Design

This was a case-control study. The level of soluble HLA-G was compared between breast cancer patients and control women. Also, the frequency of individual alleles and genotypes of +3142G/C polymorphism were compared between patients and control women. The information about metastatic status was obtained from the patients' files for determination of relationship between soluble HLA-G molecule and cancer progression. Additionally, information about ethnic groups to which subjects belong was obtained directly from the patients.

Study population, Sample size and Selection

The peripheral blood was collected from breast cancer patients admitted at the Ocean Road Cancer Institute (ORCI). The control group included women attending screening service at ORCI, diagnostically found to have no breast or cervical cancer.

According to information from Global disease burden site accessed on 22nd January, 2018 (http://global-disease-burden.healthgrove.com/l/33225/Breast-Cancer-in Tanzania#Explore%20More&s=yhT6n), the prevalence of breast cancer is 5% in Tanzania. By utilizing this information about prevalence of breast cancer, setting the confidence level and margin error at 95% and 5% respectively, the underneath formula determined the sample size as follows:

$$n = \frac{z^2 p(100 - p)}{\varepsilon^2}$$

n = Sample size

z = corresponding to the level of confidence (95%)

ε= Margin of error (5%)

p= proportion of the characteristic of interest (8%)

Plugging in the values in the formula; the sample size, n for breast cancer is 73.

The collected sample size was 75 for cases and 84 for controls.

Inclusion Criteria: All female breast cancer patients attending clinics at ORCI unless otherwise specified.

Exclusion Criteria: Pregnant women and HIV+ women. These conditions are potentially capable of confounding the results for soluble HLA-G level in subjects' bloodstream.

2.2. Variables

Histopathological parameters were obtained from patients' medical files. These included metastatic status, receptor expression status (PR, ER and HER2) and mastectomy status. To determine the association of soluble HLA-G expression and breast cancer, the level of soluble HLA-G was a dependent variable while a health status (breast cancer patients and controls) was an independent variable. Also, the level of soluble HLA-G from the plasma of patients was treated as a dependent variable and metastatic status as an independent variable in determining possible association of soluble HLA-G expression and tumor progression. The level of soluble HLA-G was determined by ELISA and the information about tumor progression of patients was obtained from their medical files.

To determine prevalence of individual alleles/genotypes of HLA-G +3142 (i.e. Alleles: +3142G and +3142C; Genotypes: +3142GG, -GC and -CC), the individual alleles and genotypes were treated as independent variables while their frequencies in both cases and controls were treated as dependent variable. To test for association between a given allele or genotype with breast cancer, the frequency of individual HLA-G +3142G/C alleles and genotypes (i.e. Alleles: +3142G and +3142C; Genotypes: +3142GG, -GC and -CC) were treated as co-variables while health status (normal or diseased) was a dependent variable, and Odd ratio was determined as a measure of association. Genotyping of +3142G/C SNP was performed by LightSNiP assay using quantitative Real-Time PCR.

2.3. Data Collection Methods

Data on clinicopathological parameters of patients were collected directly from patients' files upon being authorized by responsible personnel. Laboratory based data were recorded directly at the end of each experiment.

For soluble HLA-G, peripheral blood from individual subjects was centrifuged to obtain serum which was loaded into individual wells of 96-welled and antibody pre-coated ELISA plate (Qayee Biotechnology), followed by addition of enzyme-linked monoclonal antibody. The contents were incubated for 1 hour at 37^0C followed by washing step. After that, a chromogenic substrate was added, waited for 10 minutes before reading the optical density (OD) at 450 nm wavelength under ELISA reader. The ODs were converted into their corresponding concentrations using the ELISA analysis software obtainable at *elisaanalysis.com.*

For HLA-G 3142G/C analysis, Genomic DNA was firstly extracted from peripheral blood leukocytes by manual method using Promega reagents. DNA quantities and purity were determined by Spectrophotometer (Thermo Scientific NanoDrop™ 1000 Spectrophotometer Wilmington, Delaware USA). The DNA was stored at -20ºC before subsequent genotyping.

After having DNA extracted, genotyping of rs1063320 SNP (a reference sequence for HLA-G-+3142G/C) was done by **LightSNiP typing Assay** (TIBMolBiol, Berlin, Germany). Genotyping was done by analyzing the melting curves with the LightCycler® 480 system available from Roche Diagnostics. Real-time PCR reactions were performed in 96-well PCR plate. The assay used fluorophore labeled HybProbe custom-designed to hybridize to a specific SNP variant. The respective genotype was obtained through Melting-curve analysis program. The PCR program is summarized in Figure 1, and Figure 2 shows the melting curves of rs1063320 genotypes as observed on the computer.

Programs

Program Name	pre-incubation		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:10:00	4.40		0	0	0

Program Name	amplification		
Cycles	45	Analysis Mode	Quantification

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:10	4.40		0	0	0
60	Single	00:00:10	2.20		0	0	0
72	None	00:00:15	4.40		0	0	0

Program Name	melting curve		
Cycles	1	Analysis Mode	Melting Curves

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:30	4.40		0	0	0
40	None	00:02:00	1.50		0	0	0
75	Continuous		0.19	3	0	0	0

Program Name	cooling		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
40	None	00:00:30	1.50		0	0	0

Figure 6*: PCR program for HLA-G +3142G/C (rs1063320) genotyping by LightCycler® 480. The program comprised of four phases: one cycle of pre-incubation at 95° C; 45 cycles of amplification at 95° C (denaturation), 60° C (annealing) and 72° C (extension); one cycle of melting curve at 95° C (denaturation), 40° C (annealing), and 75° C (extension); and one cycle of cooling phase at 40° C.*

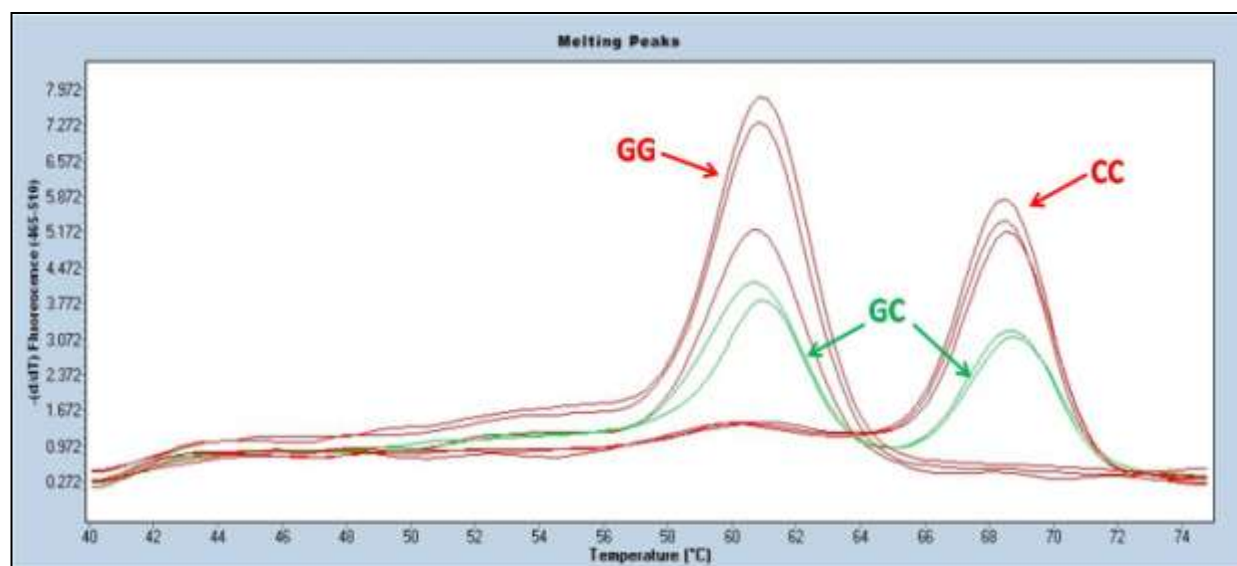


Figure 7: *Melting Curves for HLA-G +3142G/C (rs1063320) genotyping by LightCycler® 480. The genotyping relied on the difference in probe-annealing temperature to rs1063320 allelic variants-containing amplicons. The fluorescent probe hybridized to +3142C and +3142G alleles, and gave peak*

fluorescence at 68° C and 60° C respectively (red curves). Heterozygosity was unraveled by presence of two peaks, both at 68° C and 60° C (green curves).

2.4. Investigation Tools and Validity and Reliability issues

The major investigation tools used were ELISA kits and quantitative Real-Time PCR (LightCycler® 480). The validity of ELISA as a tool was determined by performing a series of dilution of a standard sample (pure sHLA-G provided by manufacturer) and reading the resulting concentration of diluted portions after immunoassay. The standard and blank controls and were run in duplicates. The ODs of standards were consistent with their concentrations. The validity of genotyping by quantitative Real-Time PCR (LightCycler® 480) was validated by running a demo experiment by using few samples and witnessing the colors and patterns of genotyping curves similar to that offered in manufacturer's instructions leaflet. Also, controls were run parallel with test samples.

Data Analysis

In this study, non-parametric tests were used. To determine whether there is an increased expression of soluble HLA-G in breast cancer patients, the comparison of plasma HLA-G between breast cancer patients and controls was statistically performed. The Mann-Whitney test was used to compare the median levels of soluble HLA-G between the groups. The analysis of sHLA-G with respect to metastatic status was also performed by using Mann-Whitney test. The comparison of plasma HLA-G across the three genotypes was done by Kruskal-Wallis test. Receiver operating characteristic (ROC) Curve; the ROC curve allows to complete sensitivity / specificity reports. The area under the ROC curve (AUROCs) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal) (www.medcalc.org). ROC analysis was used to assess the diagnostic utility of soluble HLA-G. The association was considered to be statistically significant when p-value was below 0.05. SPSS version 25 was used as statistical package software.

To determine the association between HLA-G +3142G/C polymorphism and breast cancer development, odd ratios (ORs) were determined for each individual alleles and genotypes using Hardy-Weinberg Equilibrium (HWE) software. An allele/genotype with OR less than 1 was considered to have a risk association with the diseases while with OR greater than 1 was

considered to be protective against breast cancer. The measure of association was considered to be statistically significant when the *p* value was less than 0.05.

Retrospective follow-up was conducted from the date of mastectomy to the date of last news for patients without any metastatic relapse. Metastasis-free survival (MFS) was calculated from the date of diagnosis until the date of first distant relapse using the Kaplan-Meier method. Survival was compared between groups with the log-rank test.

2.5. Ethical Issues

This study used biological samples (blood) from subjects. Before taking the sample, Swahili written informed consent was given to each subject and sample was collected only when a subject accepted signed the consent. This research was conducted after being approved by the Muhimbili University of Health and Allied Sciences (MUHAS) Ethical committee. The patients' data were handled confidentially, by making sure that names and anything that can identify them were kept in a special file accessible to the main researcher only. The confidentiality was maintained further by replacing the participant's names with identification number in course of laboratory experimentation and data collection from experiments.

2.6. Study Limitations and Mitigation

Obtaining targeted sample size from subjects in an appropriate time seemed to be challenging, for participant's consent had to be sought prior to their participation. However, through the help from nurses operating the clinics, subjects were well informed about the research and we had their consents.

It was quite challenging to obtain the information pertaining tumor stages of the participating patients. As a result, the dichotomy approach was used to classify tumor progression into metastasized and non-metastasized groups.

Majority of patients in this study were already under medication. Therefore, the study lacks the information on the effect of cancer treatment on the sHLA-G expression among breast cancer patients. However, the insight obtained from this study can still hold to be important in understanding the implication of HLA-G in breast cancer.

This study was initially meant to involve both breast and cervical cancer patients, but due to the financial constraints and limited available time to finish this dissertation, only breast cancer was

opted to be studied. Furthermore, due to budgetary constraints, DNA was extracted by manual method using Promega reagent (and not automated MagnaPure robots) and the ELISA reading was done once and not in multi-plicate as recommended by many researchers.

3. RESULTS

3.1. Clinical characteristics of study population

A total of 75 breast cancer patients with median age 49 and 84 normal control women with no clinically reported cancer (median age 43) were enrolled in this study. The median BMI of breast cancer patients was 27. The clinical characteristics of these subjects are summarized in Table 3. All breast cancer patients were under medication with either chemotherapy, radiotherapy, hormone therapy or both. About 81.3% of the patients had undergone mastectomy and the mode of post-mastectomy time was 1 year. About half of the patients had their cancer metastasized. Also, regarding receptor expression status, about half of the patients were not expressing progesterone receptors (PR) as revealed by immunohistological test. The receptor expression status of 29.3% of patients could not be obtained from their medical files. Most patients belonged to the ethnical groups found in Southern and Eastern parts of Tanzania.

Table 6: Clinical Characteristics of Breast Cancer Patients

VARIABLE	VALUE
Median Age (IQR)	49(41-60)
<median age, n (%)	37 (49.3)
≥median age, n (%)	38 (50.7)
Median BMI (IQR)	26.98 (22.67-33.65)
<median BMI, n (%)	39 (52)
≥median BMI n (%)	36 (48)
Metastatic status, n (%)	
Metastasized	39 (52)
Non-Metastasized	36 (48)
Mastectomy status, n (%)	
Yes	61 (81.3)
No	14 (18.7)
Receptor status, n (%)	
ER positive	24 (32)
ER negative	29 (38)
PR positive	16 (21.3)
PR negative	37 (49.3)
HER positive	21 (28)
HER negative	32 (42.7)
Unknown	22 (29.3)
Origin, n (%)	
North	21(28)
South	19 (25.3)
East	19(25.3)
West	6 (8)
Central	10 (13.3)

3.2. Plasma Level of Soluble HLA-G in Breast Cancer Patients and Controls

The median plasma level of soluble HLA-G in breast cancer patients (13.38) was significantly lower than that in normal controls (22.71). Non-mastectomized breast cancer patients had significantly higher plasma HLA-G than mastectomized patients (p= 0.018). No significant difference in plasma HLA-G level was found with respect to the expression status of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Table 7).

Table 7: Soluble HLA-G with respect to Clinicopathological parameters

Parameter	Mean sHLA-G level	Median sHLA-G level (IQR)	*p* value
Disease status			
Patients	23.92	13.08 (11.38-18.44)	0.000
Control	25.68	22.71 (22.29-23.77)	
Mastectomy status			
Yes	21.22	12.67 (11.29-16.30)	0.018
No	35.70	15.40 (12.80-55.25)	
Metastatic status			
Metastasized	17.14	12.93 (11.32-14.80)	0.252
Non-metastasized	30.17	13.22 (11.64-23.27)	
ER status			
Positive	26.58	12.76 (11.33-16.84)	0.611
Negative	23.00	11.77 (11.05-15.40)	
PR status			
Positive	29.91	12.32 (10.82-16.80)	0.663
Negative	22.34	12.67 (11.33-15.40)	
HER2 status			
Positive	30.82	11.44 (10.81-27.87)	0.592
Negative	20.56	12.76 (11.65-14.84)	

p values were obtained by Mann-Whitney test; Statistical test was significant if p<0.05

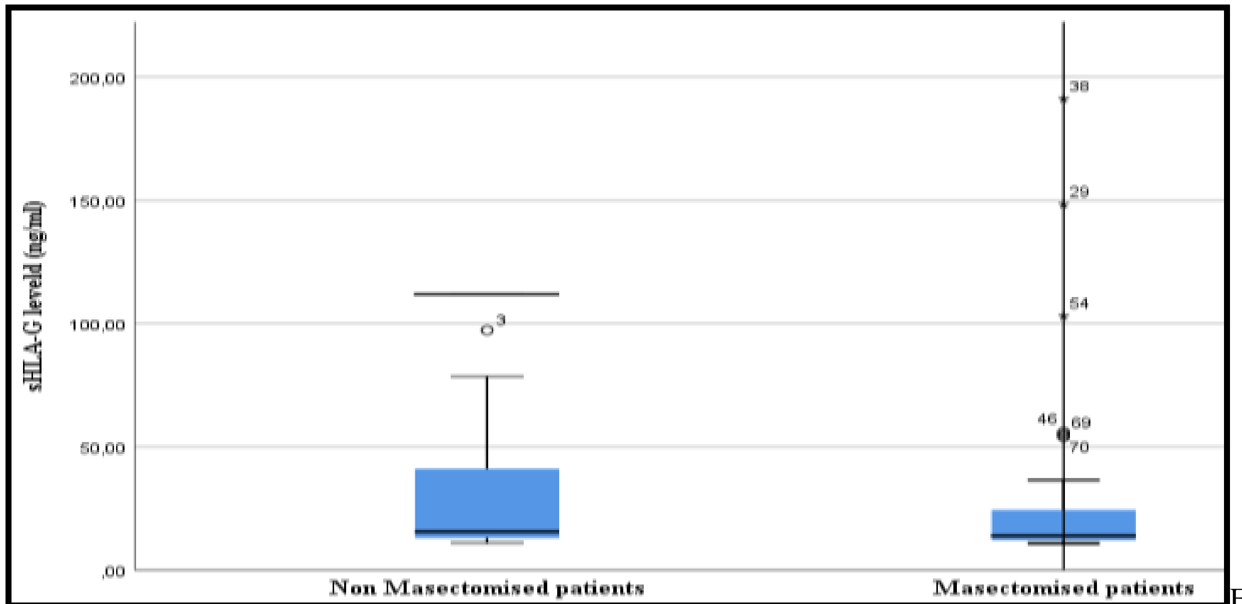


Figure 8: *Box plot presenting sHLA-G level in mastectomized and non-mastectomized breast cancer patients. Data are expressed as mean values.* *(Mann-Whitney test, p<0.001)*

3.3. Regional Comparison of sHLA-G level among Breast Cancer Patients

The levels of sHLA-G were further compared among breast cancer patients with respect to their regions of origin in Tanzania (Figure 9). There was no significant difference in sHLA-G levels observed among the regional groups (Kruskal Wallis test, p=0.756).

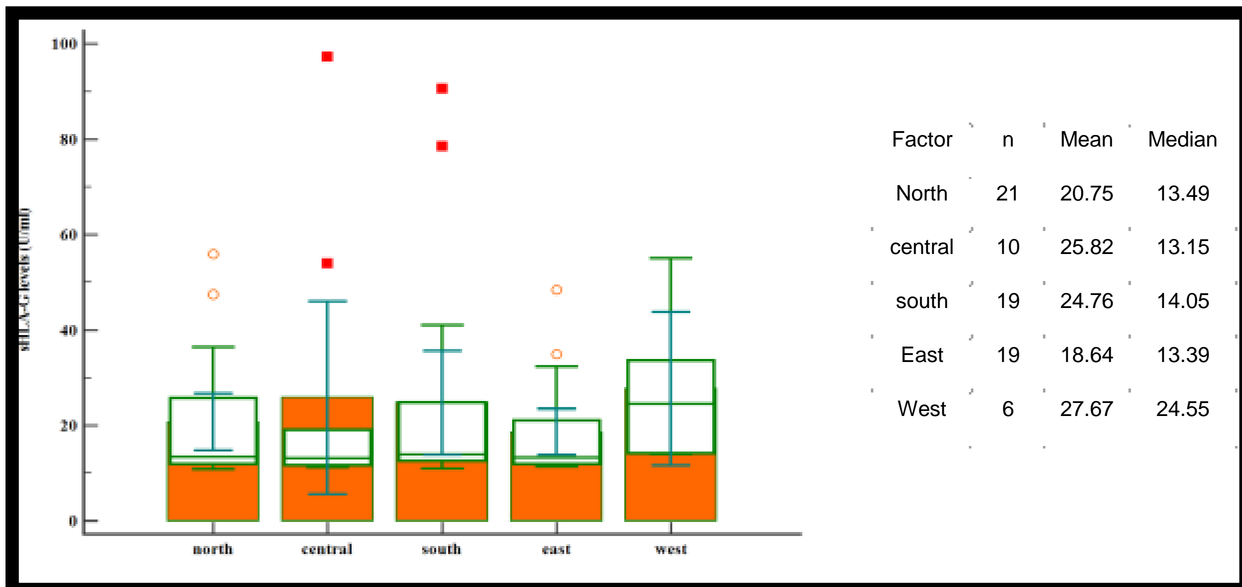


Figure 9**:** *Box plot presenting serum HLA-G levels in breast cancer patients from different regions of Tanzania (as indicated north, south, central, east and west part of Tanzania). Data are expressed as mean values. (Kruskal-Wallis test, p= 0.756).*

3.4. Frequency Distribution of HLA-G +3142G/C Genotypes and Alleles among Breast Cancer Patients and Controls

The genotype and allelic frequency distributions are summarized in Table 8. The total of 64 and 75 samples of breast cancer patients and normal controls respectively were genotyped for +3142G/C SNP. The rest samples could not be genotyped due to poor quality and low quantity of their DNA. In the whole genotyped sample population, homozygous wild-type, CC (33.9%) was more prevalent than homozygous mutant, GG (19.7%). The heterozygous genotype constituted 46.4% of the sample population.

With reference to GG genotype, there was no significant difference in genotypic frequency distributions between breast cancer patients and normal controls (p=0.537). Similarly, the allelic frequency distribution was relatively similar between breast cancer patients and controls (p=0.650). The ancestral (wild-type) allele C is considered to be associated with more expression of HLA-G than mutant G allele (174). Since the more expression of HLA-G molecules suppresses the immune function and hence increases the chance for the tumor cells to develop into malignancy, ancestral C is a risk allele and G is a protective allele. In this study, the protective GG genotype is more prevalent in control group (24.6%) than in breast cancer patients group (13.8%). However, the protective effect of GG is found to be weak, with OR=1.26 (95% C.I 0.6-2.65).

The deviation of +3142G/C genotypic distribution from Hardy Weinberg Equilibrium (HWE) was also tested. Although not statistically significant, controls had more deviation (p=0.09) than breast cancer patients group (p=0.41).

Table 8: Genotype and Allelic Frequency Distribution of +3142G/C among Breast Cancer Patients and Controls: G is a reference allele

	*Patients, (%) n=64	**Controls, (%) n=75	X^2	p-value	OR/95%CI
Genotype					
CC	18 (31%)	25 (36.2%)	0.38	0.537	1.26/0.6-2.65
GC	32 (55.2%)	27 (39.1%)			
GG	8 (13.8%)	17 (24.6%)			
Allele					
C	38 (59%)	42 (56%)	0.21	0.650	0.89/0.54-1.46
G	26 (41%)	33 (44%)			

**X^2 Fitness-of-Fit test for Deviation of allelic and genotypic frequency distribution from HWE in Patients, p=0.420; **X^2 Fitness-of-Fit test for Deviation of allelic and genotypic frequency distribution from HWE in Controls, p=0.09; OR: Odd ratio; CI: Confidence interval*

3.5. Geographical Frequency Distributions of rs1063320 Genotypes among Breast Cancer Patients

The zones thought to be the origin of ethnic groups to which breast cancer patients belonged were identified, and classified as northern, southern, eastern, western and central zones. The frequency distributions of genotypes are presented in Figure 10. Patients belonging to ethnical groups with ancestral origins found in Eastern and Central parts of Tanzania had the highest and similar prevalence of GG genotype, but GG genotype could not be detected in patients hailing from northern part of Tanzania. While CC genotype had the highest prevalence in patients hailing from Eastern part of Tanzania, this genotype had lowest prevalence in central region-ethnically tied patients. However, the frequency distributions of both genotypes across all the regions was not statistically different (p=0.157).

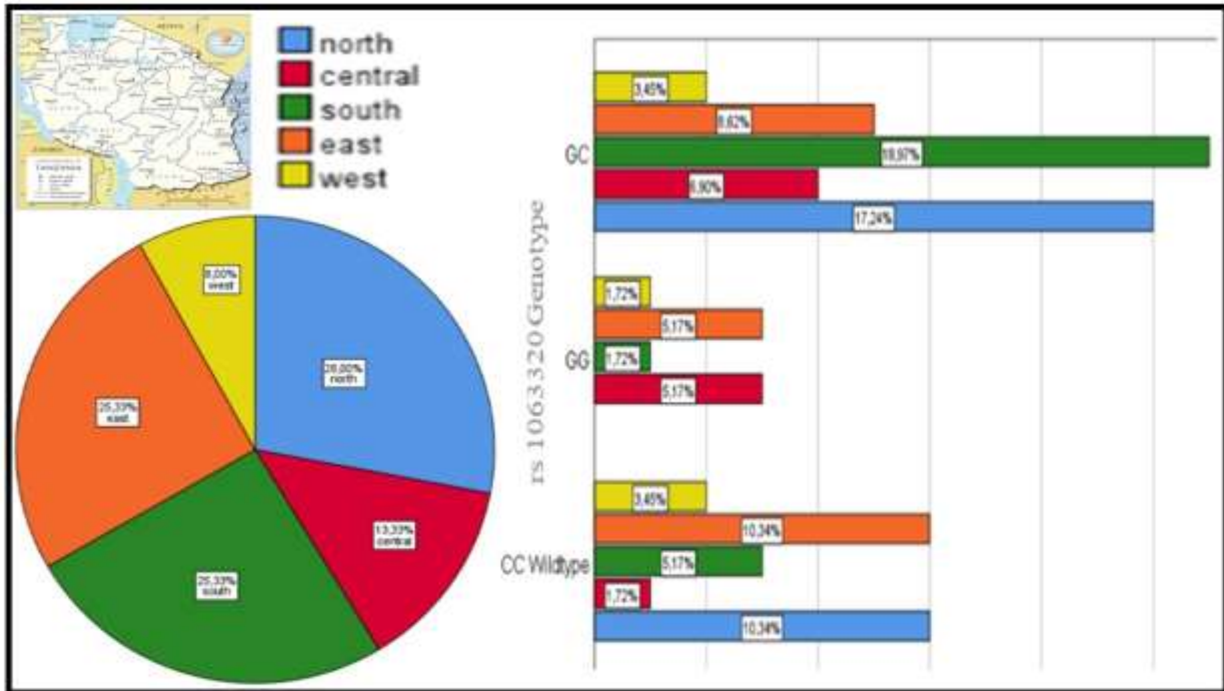


Figure 10**:** *Regional Distribution of rs1063320 Genotypes among Breast Cancer Patients at ORCI, Tanzania. No significant difference in genotype frequencies distribution among regions was found (p=0.157, Pearson-Chi-square test).*

3.6. Genotypic and Allelic Frequencies Distribution among Selected African Ethnics

The data for rs1063320 genotypic and allelic frequencies of African ethnics were extracted from HapMap and 1000 Genome project repositories. The ethnic populations data extracted from HapMap included genotype data for Luhya in Webuye from Kenya (LWK), Maasai in Kinyawa from Kenya (MKK) and people of African ancestry living in Southwest USA (ASW). Data extracted from 1000 Genomes were for Yoruba from Nigeria (YRI) and sample generalizing all African population (AFR). Their genotypic frequencies distribution patterns were compared with Tanzanian one obtained from this study (TNZ) (Table 9). The distributions of genotypic frequencies in all populations conformed to HWE. With this studied Tanzanian population as a reference, only Luhya population had a genotype and allelic distributions relatively similar to it.

Table 9: Genotypic and Allelic frequency distribution of rs1063320 among African ethnics

Population		TNZ	LWK	MKK	YRI	ASW	AFR
N		139	89	142	108	49	661
Genotype frequency (%):	CC	33.9	23.6	8.5	12.0	10.2	13.8
	CG	46.4	53.9	40.8	46.3	57.1	48.3
	GG	19.7	22.5	50.7	41.7	32.7	38.0
*p**			**0.104**	0.000	0.000	0.002	0.000
Allelic frequency: (%)	C	57	50.6	28.9	35.2	38.8	37.9
	G	43	49.4	71.1	64.8	61.2	62.1
*p***			**0.180**	0.000	0.000	0.002	0.000
*p****		0.588	0.529	1.0	1.0	0.231	0.563

Chi-square test integrated in HWE software was used for comparative analysis of genotypic and allelic frequencies distribution; p and p**: p-values for genotypic and allelic frequencies distribution (respectively) comparison between respective population and Tanzanian (TNZ); ***p: Fisher's test p-value for deviation of genotypic distribution from HWE. Association was significant if $p<0.05$.*

3.7. +3142G/C Genotypes and Plasma Levels of Soluble HLA-G

To explore whether +3142G/C genotypic variants affect the expression of HLA-G, association of +3142G/C genotypes with plasma level of soluble HLA-G was determined. The levels of sHLA-G across all genotypes were relatively similar (Kruskal Wallis test, p=0.684).

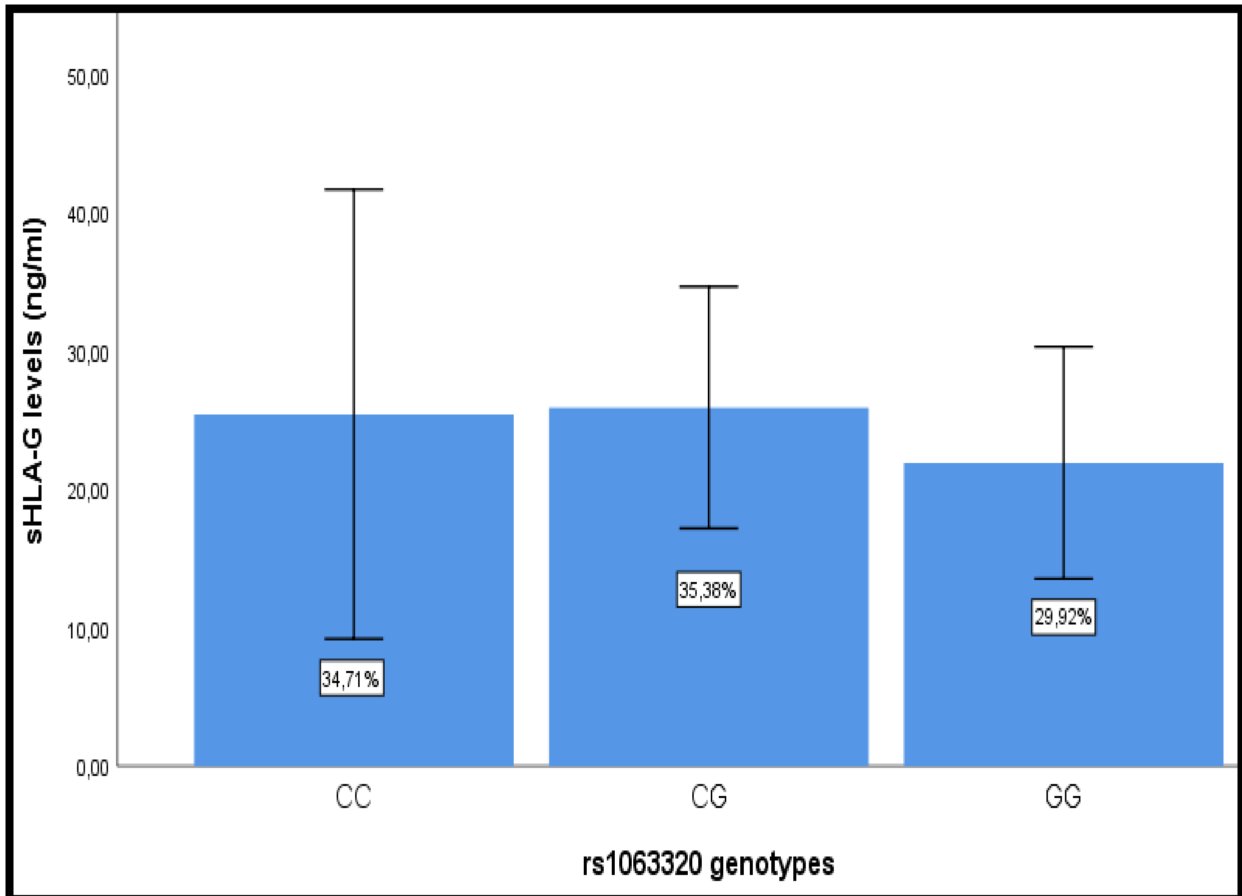


Figure 11*: Bar graph presenting genotype specific level of sHLA-G in the study population. No significant difference in sHLA-G levels between genotypes was found (Kruskal Wallis test, p=0.684)*

3.8. Predictive Power and Prognostic Value of sHLA-G Levels

The receiver operating characteristic (ROC) curve of sHLA-G in predicting breast cancer are presented in Figure 12, and pair-wise comparison between areas under ROC curves (AUCs) were performed. When breast cancer status was diagnosed by sHLA-G levels alone (Fig. 12A), it showed a statistically significant discriminatory performance ($p < 0.001$). When breast cancer was diagnosed by sHLA-G levels as well as the genotyping (CC versus CG+GG) results (Fig. 12B), the difference in AUCs showed a statistical significance ($p<0.001$). Taking mastectomy and metastasis status of patients into consideration, the predictive power of sHLA-G was determined (Figure 13A and 13B). Breast cancer patients with and without metastasis were analyzed with sHLA-G levels and did not show a statistical significance (AUC: 0 .562 (95% CI= 0,442 to 0,676, $p<0.359$), when the analysis was performed by mastectomy status, a statistical

significance was achieved (AUC: 0.667, 95 CI: 0,549 to 0,772, p=0.041) for the ability of sHLA-G to predict mastectomized patients.

Pair-wise comparison between areas under ROC curves (AUCs) of ER status, PR status, HER2 status, metastatic status, and sHLA-G were performed with respect to mastectomy status. The significant differences in AUC were found between sHLA-G level versus ER status (AUC difference: 0.228, 95% C.I: 0.0113 to 0.445, p=0.0392), and sHLA-G level versus PR status (AUC difference: 0.198, 95% CI: 0.00908 to 0.386, p=0.0399).

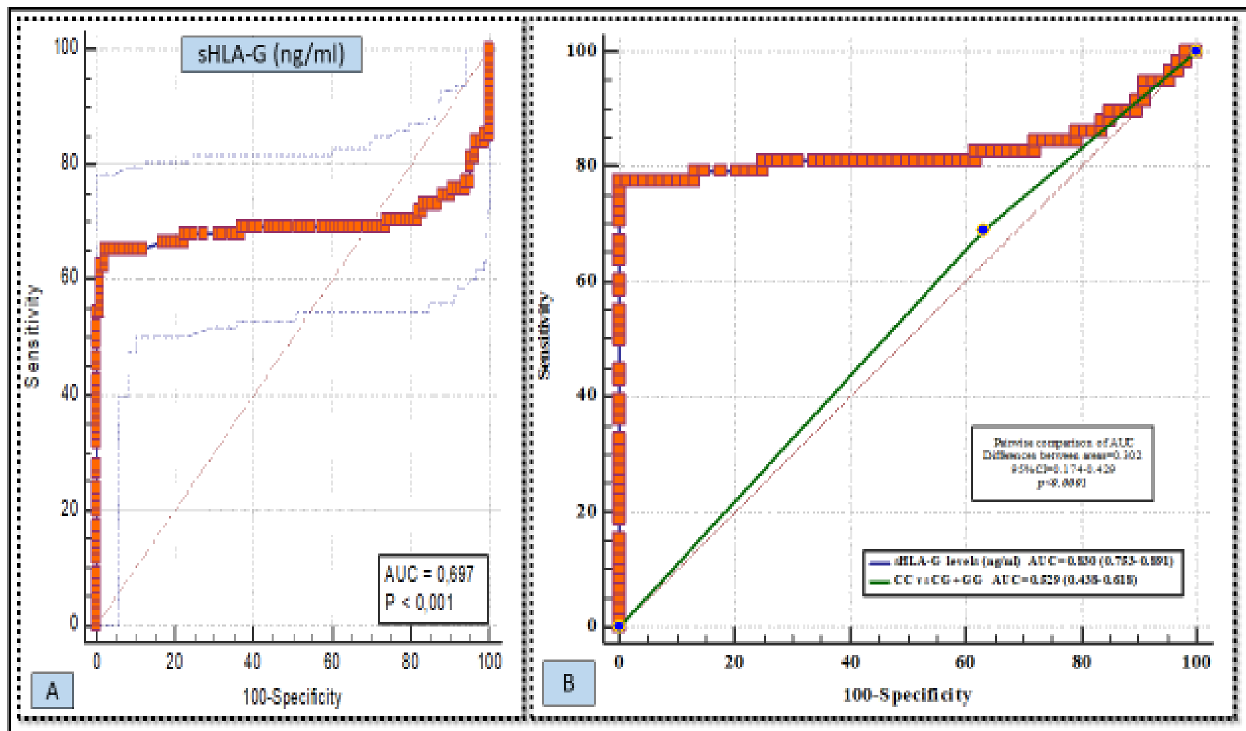


Figure 12: (A) *Receiver operating characteristics (ROC) curve analysis assessing the performance of sHLA-G in discriminating between breast cancer patients and controls. The area under curve (AUC) is 0 .697 (95% CI= 0.619-0.767, p<0.001). (B) Pair-wise comparison of the ROC curve between sHLA-G levels and CC and CG+GG genotypes in predicting breast cancer. Differences between areas is AUC=0.302 (95% CI=0.174-0.429), p<0.0001. The statistical significance of the difference between 2 ROC curves was evaluated with the method of DeLong et al.* (175).

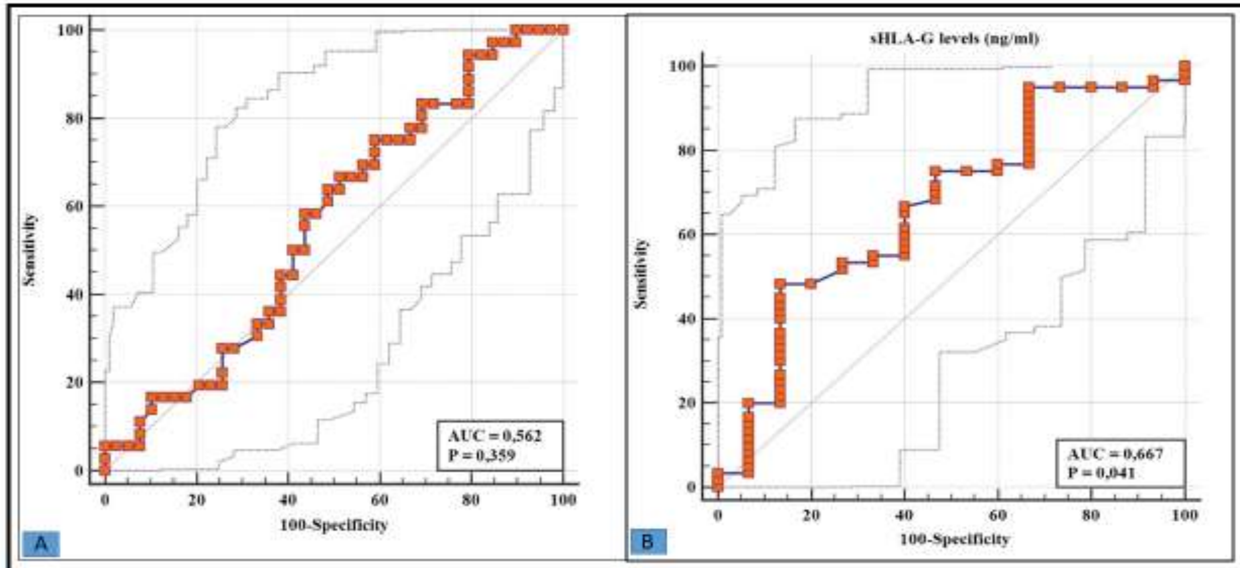


Figure 13: *Receiver operating characteristics (ROC) curve analysis assessing the performance of sHLA-G in discriminating between A: metastatic and non-metastatic breast cancer patients and B: mastectomized and non-mastectomized breast cancer patients. The area under curve (AUC) is 0 .562 (95% CI= 0.442 to 0.676, p<0.359) and 0.667 (95 CI: 0.549 to 0.772, p=0.041) respectively. The statistical significance of the difference between 2 ROC curves was evaluated with the method of DeLong et al.* (175).

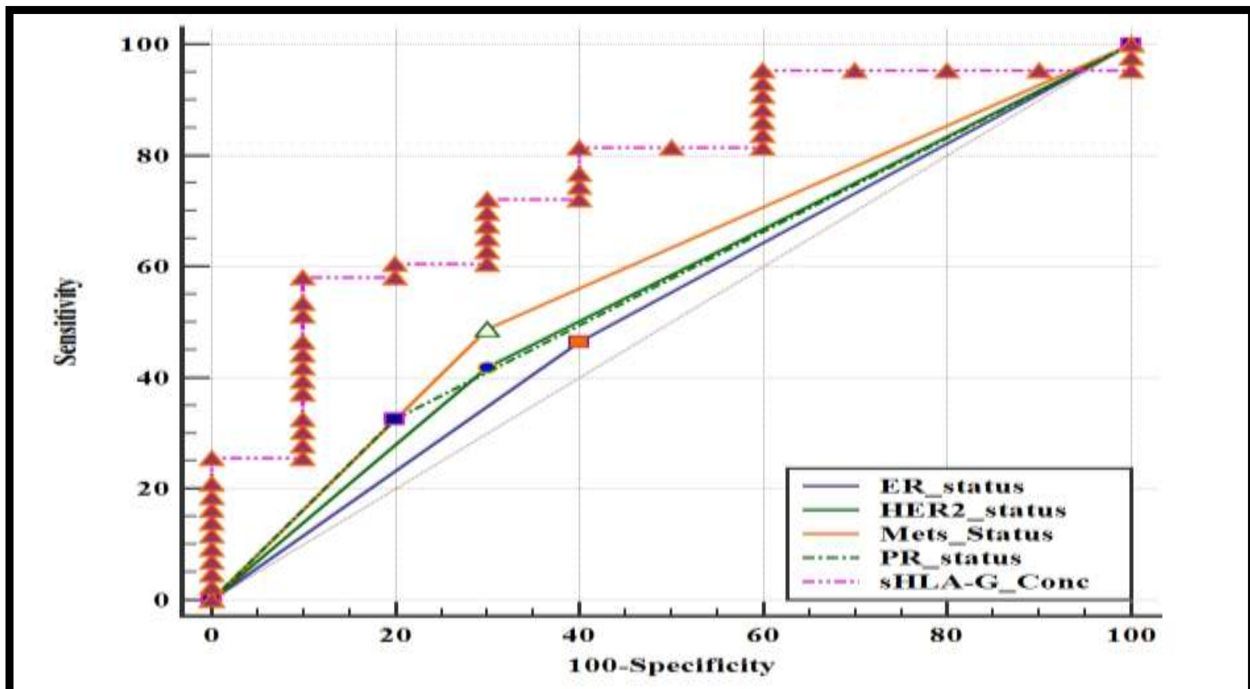


Figure 14*:Pair-wise analysis of studied variables (ER status, HER2 status, PR status, Metastatic status and sHLA-G level) to discriminate between mastectomized and non-mastectomized breast cancer patients. Significant differences in ROC AUCs were observed between sHLA-G level versus ER*

expression status (0.228, 95% C.I: 0.0113 to 0.445, p=0.0392) and sHLA-G versus PR expression status (: 0.198, 95% CI: 0.00908 to 0.386, p=0.0399).

3.9. Kaplan-Meier Analysis of Metastasis-free Breast Cancer Survival

The power of +3142G/C genetic variants to predict the metastatic-free survival among breast cancer patients was investigated by comparing patients carrying no risk allele (GG) versus those carrying at least one risk allele (CC and CG). The patients were retrospectively followed up from the date they had mastectomy to the date of last news for patients without any metastatic relapse. Metastasis-free survival (MFS) was calculated from the date of diagnosis until the date of first distant relapse using the Kaplan-Meier method. Survival was compared between groups with the log-rank test. The longest follow up time was 12 years. In both groups, most metastatic relapses occurred within 5 years following mastectomy. No significant difference in MFS was found between the two groups (Log rank, p=0.6508). However, further studies with more clinical records and increased patients sample size are needed to reach a solid conclusion.

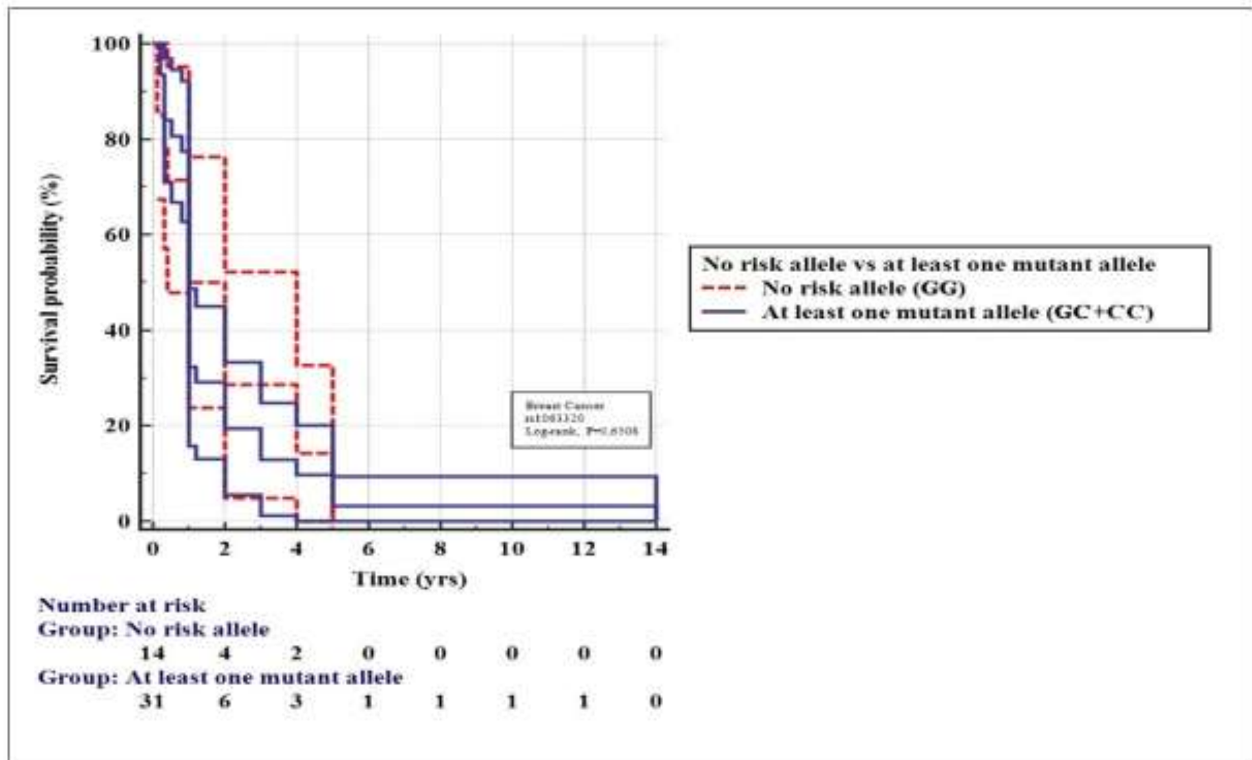


Figure 15*: Kaplan-Meier analysis of metastasis-free survival in breast cancer patients with mastectomy for rs1063320 genotypes. Comparison of MFS curves for patients with at least one risk allele (C) and no risk alleles (GG for rs1063320): in breast cancer. No significance difference was found (p=0.6508).*

4. DISCUSSION

The discovery of HLA-G molecule as a potential mediator of maternal-fetal immune tolerance in trophoblast cells intrigued many researchers to extrapolate those findings in tumor immunology. Both trophoblasts and tumor cells express low level or no classical HLA-1 molecules, plausibly as a strategy to avoid their recognition by T cells. All nucleated cells express classical HLA-1 molecules and downregulation of all or some of these molecules puts in jeopardy the cell to be susceptible to NK cytolytic attack (176). The ability of tumor cells with downregulated level of classical HLA-1 to make their way to malignancy without being destroyed by NK cells implies that some strategy must be employed by these tumor cells preventing their recognition and destruction by NK cells. HLA-G is thought to be a molecule that suppress this NK response to potentially susceptible tumor cells (177).

Considerable number of studies has demonstrated the immunosuppressive effect of HLA-G in both in vitro and in vivo. The in vitro study by Rouas-Freiss*et al.* (18) investigating the protective role of HLA-G against NK cytolytic effect showed that HLA-G bearing trophoblasts could not be lysed by semi-allogeneic or allogeneic NK cells, and this protection was inhibited by anti-HLA-G mAbs. The in vivo study by Loumagne *et al*. utilized tumor lines (4T1 and FON-), some transfected with hβ2m only and others transfected with both hβ2m and HLA-G5. Upon injecting these transfected cells into immunocompetent mice, tumor cells not transfected with HLA-G5 were rejected by mice immune system, while those transfected with HLA-G5 developed into malignancy. These findings suggest the pro-tumorigenic influence of HLA-G molecule (17).

Some research findings however impose some cautions on generalizing the inhibitory role of HLA-G to immune response. HLA-E has been found to have similar immunosuppressive effect (178), and its expression is upregulated by expression of HLA-G. The upregulation is achieved at proteomic level at which the leader sequence of HLA-G, which is not incorporated in final HLA-G protein product is cleaved and binds to and stabilizes the HLA-E protein (179). It is possible that HLA-G mediates immunosuppression indirectly through HLA-E molecule. Moreover, the recently in vitro study by Veluchamy*et al*. showed that the efficacy of NK cells derived from umbilical cord and peripheral blood to lyse the cervical cancer cell lines was independent to neither HLA-G nor HLA-E. (139). Gao *et al*. also reported that classical HLA-1 molecules and

not HLA-G in myeloma cells confer an inhibitory effect to NK cytoxicity (158). All these findings boil down to the possibility that HLA-G expression may not be significantly involved in the process of carcinogenesis.

Increased expression of surface and soluble HLA-G in breast cancer and some other cancer patients has been reported by many researchers, and various cut-off point values have been suggested by these researchers for utility of this molecule as a diagnostic marker (Table 5). Furthermore, sufficient research show that surface and soluble HLA-G expression increases with cancer progression (27,33,35) and call for this molecule to be used as a prognostic marker.

In this study, the results show a significantly higher level of sHLA-G in normal controls than in breast cancer patients. Although contrary to what most studies report, there exists a number of research works in line with these findings. Some of such researches show the irrelevance of HLA-G expression in cancer development. But, others acknowledge the upregulation of HLA-G in course of cancer development and add that medical intervention such as chemotherapy, mastectomy and adjuvant treatment lowers the expression level of HLA-G.

The study by Real *et al*. characterized the expression of HLA-G in 50 tumor tissues and some tumor cell lines at mRNA and protein level. Although some HLA-G mRNA of variety forms could be detected in the tissues and cell lines, no evidence of HLA-G protein detection was found in tumor tissues. HLA-G protein tested positive in myelomonocytic cell lines only after being treated with interferon-gamma, and the researcher concluded by making a remark that HLA-G expression in tumors is an infrequent event (156). Similar findings were found by Frumento *et al.* (157) in melanoma tissues and cell lines.

The investigation by Pangault *et al.* (155) in liver, colon, breast, kidney, ovary, and larynx derived malignant cells along with some tumor cell lines could not find the expression of HLA-G protein in most tissues, except in two tumor cell lines (JEG3 and MCF-7). Although this study reported HLA-G expression in activated tumor infiltrating macrophage, the following study by Davies B*et al.* (154) could not find the evidence for HLA-G expression in neither tumor tissues nor in any type of tumor infiltrating leukocytes.

The aforementioned studies investigated the expression of surface bound forms, and not soluble forms of HLA-G protein in tumor tissues and cell lines. Therefore, the possibility of the detected

HLA-G mRNA to code for soluble HLA-G protein cannot be ruled out. However, the study conducted by Akin *et al*. among thyroid cancer patients gave corroborative results to this study's findings. The comparison of plasma level of soluble HLA-G between thyroid cancer patients and normal healthy individuals revealed that thyroid cancer patients had a significantly lower level of plasma HLA-G than healthy individuals (180). Also, the level of plasma HLA-G in neuroblastoma patients was reported to be similar to that in normal control group by Morandi *et al.* (40). On top of that, the study by Gan *et al*. found that the level of plasma soluble HLA-G in bladder transitional cell carcinoma patients was not significantly different from that in normal controls (163).

However, it should be recalled that most of the patients enrolled in this study were already under medication, both chemotherapy and radiotherapy or hormone therapy, and 81.3% had undergone mastectomy. It is plausible to think of how these clinical characteristics may profoundly influence the bioavailability of HLA-G in the circulation. Since the elevated plasma level of HLA-G in cancer patients is mostly attributed to its increased expression by tumor cells, surgical removal of these HLA-G expressing cells is expected to lower its bioavailability. In this study, the plasma sHLA-G level was significantly lower in mastectomized breast cancer patients than in non-mastectomized patients, and ROC analysis revealed a good performance of sHLA-G to discriminate between mastectomized and non-mastectomized breast cancer patients.

The plausible influence of clinical characteristics pinpointed above is in agreement with the findings obtained from the follow up study conducted by Sayed *et al*. This study clearly shows that mastectomy and adjuvant therapy in breast cancer patients significantly lower the plasma sHLA-G level. In this study, the mean level of sHLA-G in patients before surgery (mean sHLA-G =50.68) was significantly higher than that in normal controls (mean sHLA-G=19.5). However, after 6 and 12 months following surgery and while under adjuvant therapy, mean sHLA-G levels significantly decreased to 13.15ng/ml and 9.8ng/ml respectively. After 6 and 12 months post-surgery, 66.7% and 97.8% of patients respectively had sHLA-G levels below normal controls' sHLA-G mean (38).

The mode of post-mastectomy time in this study population was 1 year (12 months) and is equivalent to the post-surgery time in Sayed *et al*. (38) study in which 97.8% of patients had their sHLA-G levels below mean sHLA-G level observed in normal controls. This can

apparently explain why in this study the level of sHLA-G was significantly lower in breast cancer patients than in normal controls. Therefore, our findings that sHLA-G level in mastectomized patients is significantly lower than that in non-mastectomized patients, coupled with this post-mastectomy time dependent decrease in sHLA-G level observed in Sayed *et al.* study suggests that sHLA-G can be used as a biomarker for monitoring the breast cancer patients' progress while under medication.

Some studies have already reported the effect of chemotherapy on plasma HLA-G level. Rutten *et al*. found that chemotherapy significantly lowers the plasma level of soluble HLA-G among high grade ovarian cancer patients (167). Estradiol and progesterone have been demonstrated to upregulate the expression of HLA-G. Their antagonists, tamoxifen and RU468, which are conventional hormonal receptor based therapeutics have also been found to downregulate the expression of HLA-G at least in vitro (30). This observation can be partly supported at genetic level by the fact that promoter region of HLA-G gene contains a response element for progesterone which is involved in regulation of HLA-G expression. Therefore, progesterone is among the transcriptional factors for activating HLA-G expression, and inhibiting its binding to its cognate receptor can contribute to slowing down the expression of HLA-G (181).

Furthermore, the immune cells such as monocytes and NK cells are also said to be soluble HLA-G producers (135,145,182), and so the immune cells-killing properties of radiotherapy might contribute to the decreased HLA-G bioavailability. All these clinical characteristics (mastectomy, chemotherapy, hormonal therapy and radiotherapy) in this studied breast cancer population might have had a profound impact in lowering the plasma HLA-G to the level below that observed in normal controls.

This study also shows that sHLA-G is not significantly associated with breast cancer progression and most clinical-pathological parameters studied. The sHLA-G in metastasized breast cancer patients was relatively similar to that in non-metastasized breast cancer group. Relatively similar but seemingly odd findings were reported by Swets *et al*. in the study that determined the changes in classical HLA-1 and HLA-G molecules following metastasis of colorectal cancer to liver cancer. While the changes in classical HLA-1 molecules was not evident, the findings reported decreased expression of HLA-G protein following metastasis (147). Chen *et al.* (37) and Pan *et al.* (36) also found no association between plasma level of soluble HLA-G and tumor

stage. Moreover, congruent to this study's results, lack of associations between expressions of ER, PR and HER receptors has been reported in some other research findings (37,127).

There exists variability in reported physiological levels of sHLA-G as found in normal controls in different populations. The mean physiological level of sHLA-G in women from this study's population was 25.68ng/ml. This is somewhat higher than mean sHLA-G levels 20.7ng/ml, 19.5 ng/ml, 18ng/ml and 16.23ng/ml reported by Rebman *et al* (54), Sayed *et al* (38), Septi *et al* (161) and Gros *et al* (183) respectively. Yet, the mean physiological sHLA-G level from this study's population is lower than mean levels 38.6ng/ml and 93ng/ml reported by Rudstein-Svetlicky *et al* (184) and Yie *et al* (185) respectively. This variability in sHLA-G levels may be a result of variation in standards essential for calibrating test assay (38) or merely population-wise difference in HLA-G expression. Compounded by the abnormal expression of HLA-G in some other pathological conditions such as in viral infections and autoimmune diseases, implementing the diagnostic utility of sHLA-G for breast cancer and other cancers in clinical settings becomes quite more challenging.

Post-transcriptional regulation seems to play important role in HLA-G expression. A number of studies identified HLA-G mRNA in tumor tissues and tumor cell lines without detecting the corresponding proteins (154,156,186). Some polymorphisms residing in 3' UTR region of HLA-G gene are thought to affect the stability of transcribed HLA-G mRNA. Of note, is +3142G/C polymorphism which affect the stability of HLA-G mRNA via modifying the affinity of mRNA to miRNAs.

Binding of miRNA to the mRNA leads to the recruitment of some proteins that ultimately cleave the mRNA enzymatically or prevents its translation. The G allele is considered to have high affinity to the miRNAs than +3142C alleles (179). Thus, C allele mRNA is apparently more stable and more likely to make its way to translation than G allele mRNA. Because HLA-G is considered to be pro-tumorigenic immunosuppressive molecule, the C allele which is potent to produce more HLA-G protein plausibly should be associated with cancer susceptibility. Therefore, C is a risk allele and C/C is a risk genotype.

There have been some conflicting reports on association between +3142G/C genotypes and susceptibility to cancer. Zidi *et al*. reported that genotype GG is protective against breast cancer,

consistent to what was expected from genetics grounds of HLA-G expression (127). The contrary results were reported by Zambra *et al*. in prostate cancer in which +3142G allele was tagged as risk allele (46). Garziera *et al*. found no association between genotypes or alleles of +3142G/C SNP and colorectal cancer (47). Although in this study the risk genotype CC was more prevalent in breast cancer patients than in control, no significance in association between the +3142G/C genotypes and breast cancer could be confirmed. Nor, the genetic variation at +3142G/C of HLA-G gene could not predict the metastatic-free survival in the studied breast cancer patients.

The cause for inconsistency in association of SNPs present in 3'UTR of HLA-G gene with a given trait or pathological condition across different populations has been suggested by Sabbagh *et al*. in their extensive work which characterized variations at 3'UTR of HLA-G gene at a global level. Their findings showed the different patterns of linkage disequilibrium (LD) between the allelic variants in 3'UTR across different populations worldwide. This difference could be the basis for the existing controversy over the association studies looking for HLA-G 3'UTR allelic variants and specific physiological or pathological conditions (130). Squaring with this, the significant differences in allelic and genotypic frequencies distributions were observed when our study population was compared with most populations' rs1063320 data from HapMap and 1000 Genomes.

Furthermore, the expected influence of +3142C/G alleles on sHLA-G level was not confirmed in this study as the mean and median levels of sHLA-G across all genotypes was relatively similar. This may be attributed to the complex regulatory nature of HLA-G expression, involving various environmental, transcriptional and genetic factors. Genetically, many polymorphisms other than +3142G/C such as 14bp Indel, +3027C/A +3142C/G +3003T/C, +3010C/G, +3035C/T, +3187A/G and +3196C/G (42), and many others in the promoter region (107) affect the expression of HLA-G. It is therefore important to study for haplotype specific associations, rather than individual SNPs at this locus.

It has been postulated that high nucleotide variability in 3'UTR region of HLA-G is the result of balancing selection. This has been confirmed by evolutionary insight derived from the work of Sabbagh *et al.* through exhaustive characterization of worldwide patterns of variation in

nucleotide sequence, linkage disequilibrium and haplotypes at 3'UTR among 21 populations representing Africa, America, Europe and Asia. The hallmarks for possible influence of balancing selection to the genetic structure at this locus included high diversity of nucleotide sequence, polymorphism being excess to divergence levels, low genetic differentiation across populations and existence of two haplotypes maintained at high frequencies in all populations (130).

UTR-1 and UTR-2 are the two 3'UTR haplotypes of HLA-G gene homogenously distributed in all populations and maintained at high frequencies worldwide, with relatively similar frequencies, around 25% globally. These two haplotypes differ at five variation sites; 14bp Indel, +3142G/C, +3187G/A, +3010C/G and +3196C/G (130). UTR-1 is considered to be a high-producing haplotype, normally constituted by allelic variants associated with high expression of HLA-G such as 14bp del, +3142C and +3187G. UTR-2 is a low-producing haplotype normally carrying 14bp In, +3142G and +3187A which are associated with low expression of HLA-G (42,107).

Evolutionary forces maintained both high-producing (UTR-1) and low-producing (UTR-2) haplotypes plausibly to balance the beneficial and detrimental effects associated with expression of HLA-G. The beneficial effects include protection of developing fetus from maternal immune attack, prevention of pregnancy associated complications such as miscarriage and eclampsia, and mitigation of autoimmune diseases. And the detrimental effects include immune system impairment to effectively eliminate infectious pathogens and tumors from the body (107,130).

The comparison of +3142G/C genotype distribution of our study population and other African related populations retrieved from HapMap and 1000 Genomes supports this evolutionary implication of HLA-G variation at 3'UTR region. Heterozygosity of +3142G/C was high in all populations, which ensures the maintenance of both high producing (+3142C) and low producing (+3142G) alleles in the population.

Furthermore, the possible role of balancing selection at this locus is supported by the results of HWE goodness-of-fit test from this study. The multiple allelic variants maintained in the population as a result of balancing selection normally shape the genotypic frequency distributions not to deviate from HWE. The genotypic frequency distributions at +3142G/C locus

in both our study population and the rest populations whose data were retrieved from 1000 Genomes and HapMap showed a good conformity to HWE.

The genotype and allelic frequency distribution in our study population was relatively similar to the distribution in neighboring Luhya population in Webuye from Kenya (LWK). However, the rest populations had genotypic and allelic frequency distributions significantly different from our study population. This difference in frequency distribution of allelic variants and respective genotypes can be supported by the observation by Sabbagh *et al*. (130), who reported the variation in LD patterns among the allelic variants in 3'UTR region of HLA-G gene across 21 populations worldwide.

The key limitation of this study is the lack of sufficient number of breast cancer patients who were neither under medication nor undergone mastectomy. The inclusion of this group could be crucial in determining unequivocally whether the unexpected low sHLA-G level observed in breast cancer patients is attributable to these medical interventions or not. Despite this weakness, the existing evidence on HLA-G downregulating effect of mastectomy, chemotherapy or hormone therapy somehow validates these findings. The sHLA-G lowering effect of mastectomy is further supported by our findings, as sHLA-G levels in mastectomized patients were significantly lower than that in non-mastectomized patients. Before we can fully attribute this unexpected level of sHLA-G in breast cancer patients to medical intervention, more studies consisting sufficient number of patients who have not yet started medication are emphasized.

5. CONCLUSION AND RECOMMENDATIONS

Conclusions

The Breast cancer affects women at any age. In this study breast cancer patients were younger than 83 years old and the most frequent age was 40. The youngest patient was 30 years old. On assessing the sHLA-G, the results showed that breast cancer patients had significantly lower level of sHLA-G compared to normal controls. Furthermore, the AU-ROC curve was 69.7% (95% CI; p< 0.001), which means that the test discriminates between normal individuals and patients, and sHLA-G can predict 69.7% of the patients. The AUROC analysis of sHLA-G level with respect to metastatic status was 56.2% (95% CI; p>0.05). But, the AUROC was 66.7% (95% CI; p<0.05) with respect to mastectomy status, implying that sHLA-G level can discriminate mastectomized patients from non-mastectomized patients. Taken together, the results revealed the difference in sHLA-G levels between breast cancer patients and normal controls, and between mastectomized breast cancer patients and non-mastectomized patients. This suggests its potential utility in breast cancer diagnosis and prognosis.

On the other hand, assessment of +3142G/C polymorphism revealed a relatively similar distribution of genotypes and alleles between breast cancer patients and normal controls (95% CI, p>0.05). Also, sHLA-G levels were relatively similar across CC, GC and CC genotypes of this locus (95% CI, p>0.05). The Kaplan-Meir analysis revealed no significant difference between patients carrying at least one risk allele (CC +CG) and no risk allele (GG) in their power to predict MFS (Log rank, p=0.6508). When the genotypic and allelic distribution pattern of this locus in this study population was compared to other five African populations, only Luhya population from Kenya showed relative similar distribution pattern (95% CI, p>0.05), while others had a significantly different pattern of allelic and genotypic distributions (95% CI, p<0.01). This revealed that, +3142G/C is not significantly associated with breast cancer, and the genotypic and allelic frequencies distribution of +3142G/C SNP in our study population is significantly different from the most other African related populations.

Recommendations

In light of this study results, the following are recommended: Encourage Tanzanian women to have breast exams, including self-exams and mammogram on a regular basis to enable early

prediction of any tumor at early stages for better prognosis. To carry out a wider research with sHLA-G analysis among all women who have breast cancer to provide evidence on sHLA-G as a tumor marker, and to explore more about HLA-G polymorphisms so as to unravel HLA-G polymorphic variants particularly haplotypes which can be used as genetic risk factors of breast cancer.

Most breast cancer patients presenting at ORCI had their cancers in advanced stages. This reflects that the screening programs meant for early detection of breast cancer are not much effective. This should be a call for Ministry of Health to strengthen their efforts in promoting and facilitating breast cancer screening services. Also, the ministry should provide a sufficient financial support for research looking for variety of biomarkers that can be used to detect breast cancer in early stages and as the indicators of drug response in our population. On top of that, the ministry should provide assistance in strengthening the facilities essential for breast cancer testing, especially genetic tests and related research.

The genetic tests in breast cancer prediction hold to be important in saving lives. This is because such genetic information serves a precautionary role, compelling a susceptible person to avoid as much environmental risk factors as possible and choose the lifestyle incompatible to risk aggregation. Therefore, such genetic tests are highly recommended.

6. REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-tieulent J, Jemal A. Global Cancer Statistics, 2012. CA a cancer J Clin [Internet]. 2015;65(2):87–108.
2. Denny LA, Sankaranarayanan R, De Vuyst H, Kim JJ, Adefuye PO, Alemany L, et al. Recommendations for Cervical Cancer Prevention in Sub-Saharan Africa. Vaccine [Internet]. 2013;31(S5):F73–4.
3. Liu E, McCree R, Mtisi E, Fawzi WW, Aris E, Lema IA, et al. Prevalence and risk factors of cervical squamous intraepithelial lesions among HIV-infected women in Dar es Salaam, Tanzania. Int J STD AIDS [Internet]. 2016;27(3):219–25.
4. Burson AM, Soliman AS, Ngoma TA, Mwaiselage J, Ogweyo P, Eissa MS, et al. Clinical and epidemiologic profile of breast cancer in Tanzania. Breast Dis. 2010;31(1):33–41.
5. Mayor S. Service review: improving breast cancer care in Tanzania. Lancet Oncol [Internet]. 2017;2045(17):30160.
6. Marcus A, Gowen BG, Thompson TW, Iannello A, Ardolino M, Deng W, et al. Recognition of tumors by the innate immune system and natural killer cells. Adv Immunol. 2014;122:91–128.
7. Sánchez-Paulete AR, Teijeira A, Cueto FJ, Garasa S, Pérez-Gracia JL, Sánchez-Arráez A, et al. Antigen cross-presentation and T-cell cross-priming in cancer immunology and immunotherapy. Vol. 28, Annals of Oncology. 2017. p. xii44-xii55.
8. Ishibashi K, Kumai T, Ohkuri T, Kosaka A, Nagato T, Hirata Y, et al. Epigenetic modification augments the immunogenicity of human leukocyte antigen G serving as a tumor antigen for T cell-based immunotherapy. Oncoimmunology. 2016;5(6).
9. Wu MZ, Cheng WC, Chen SF, Nieh S, O'Connor C, Liu CL, et al. MiR-25/93 mediates hypoxia-induced immunosuppression by repressing cGAS. Nat Cell Biol. 2017;19(10):1286–96.
10. Chouaib S, Noman MZ, Kosmatopoulos K, Curran MA. Hypoxic stress: Obstacles and opportunities for innovative immunotherapy of cancer. Oncogene [Internet]. 2017;36(4):439–45.
11. Wu D, Kuiaste I, Moreau P, Carosella E, Yotnda P. Rescuing lymphocytes from HLA-G immunosuppressive effects mediated by the tumor microenvironment. Oncotarget [Internet]. 2015;6(35):37385–97.

12. Renner K, Singer K, Koehl GE, Geissler EK, Peter K, Siska PJ, et al. Metabolic hallmarks of tumor and immune cells in the tumor microenvironment. Vol. 8, Frontiers in Immunology. 2017. p. 1–11.
13. Algarra I, García-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: Implications for tumor immune escape. In: Cancer Immunology, Immunotherapy. 2004. p. 904–10.
14. Corbet C, Feron O. Tumour acidosis: From the passenger to the driver's seat. Vol. 17, Nature Reviews Cancer. 2017. p. 577–93.
15. Johansen LL, Lock-Andersen J, Hviid TVF. The Pathophysiological Impact of HLA Class Ia and HLA-G Expression and Regulatory T Cells in Malignant Melanoma: A Review. Vol. 2016, Journal of Immunology Research. 2016.
16. Naji A, Menier C, Morandi F, Agaugue S, Maki G, Ferretti E, et al. Binding of HLA-G to ITIM-Bearing Ig-like Transcript 2 Receptor Suppresses B Cell Responses. J Immunol [Internet]. 2014;192(4):1536–46.
17. Loumagne L, Baudhuin J, Favier B, Montespan F, Carosella ED, Rouas-Freiss N. In vivo evidence that secretion of HLA-G by immunogenic tumor cells allows their evasion from immunosurveillance. Int J Cancer. 2014;135(9):2107–17.
18. Rouas-Freiss N, Goncalves RM-B, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc Natl Acad Sci [Internet]. 1997;94(21):11520–5.
19. Rodríguez JA. HLA-mediated tumor escape mechanisms that may impair immunotherapy clinical outcomes via T-cell activation (Review). Vol. 14, Oncology Letters. 2017. p. 4415–27.
20. Ozet G, Falay M, Dagdas S, Ceran F. Determination of HLA-G Expression and Evaluation of Its Role as a Prognostic Factor in Chronic Lymphocytic Leukemia. J Clin Lab Anal. 2016;30(5):399–403.
21. Le Discorde M, Moreau P, Sabatier P, Legeais JM, Carosella ED. Expression of HLA-G in Human Cornea, an Immune-Privileged Tissue. In: Human Immunology. 2003. p. 1039–44.

22. Morandi F, Rizzo R, Fainardi E, Rouas-Freiss N, Pistoia V. Recent advances in our understanding of HLA-G biology: Lessons from a wide spectrum of human diseases. Vol. 2016, Journal of Immunology Research. Hindawi Publishing Corporation; 2016.
23. González Á, Rebmann V, Lemaoult J, Horn PA, Carosella ED, Alegre E. The immunosuppressive molecule HLA-G and its clinical implications. Vol. 49, Critical Reviews in Clinical Laboratory Sciences. 2012. p. 63–84.
24. LeMaoult J. HLA-G up-regulates ILT2, ILT3, ILT4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells. FASEB J [Internet]. 2005;23(18):1–23.
25. Wan R, Wang ZW, Li H, Peng XD, Liu GY, Ou JM, et al. Human Leukocyte Antigen-G Inhibits the Anti-Tumor Effect of Natural Killer Cells via Immunoglobulin-Like Transcript 2 in Gastric Cancer. Cell Physiol Biochem. 2018;44(5):1828–41.
26. Zhang Y, Zhao J, Qiu L, Zhang P, Li J, Yang D, et al. Co-expression of ILT4/HLA-G in human non-small cell lung cancer correlates with poor prognosis and ILT4-HLA-G interaction activates ERK signaling. Tumor Biol. 2016;37(8):11187–98.
27. Zheng N, Wang CX, Zhang X, Du LT, Zhang J, Kan SF, et al. Up-regulation of HLA-G expression in cervical premalignant and malignant lesions. Tissue Antigens. 2011;77(3):218–24.
28. Ye SR, Yang H, Li K, Dong DD, Lin XM, Yie SM. Human leukocyte antigen G expression: As a significant prognostic indicator for patients with colorectal cancer. Mod Pathol. 2007;20(3):375–83.
29. Costa Arantes DA, Gonçalves AS, Jham BC, Duarte ECB, de Paula ÉC, de Paula HM, et al. Evaluation of HLA-G, HLA-E, and PD-L1 proteins in oral osteosarcomas. Oral Surg Oral Med Oral Pathol Oral Radiol [Internet]. 2017;123(6):e188–96.
30. He X, Dong D, Yie S, Yang H, Cao M, Ye S, et al. HLA-G Expression in Human Breast Cancer: Implications for Diagnosis and Prognosis, and Effect on Allocytotoxic Lymphocyte Response After Hormone Treatment In Vitro. Ann Surg Oncol [Internet]. 2010;17(5):1459–69.
31. Li XJ, Zhang X, Lin A, Ruan YY, Yan WH. Human leukocyte antigen-G (HLA-G) expression in cervical cancer lesions is associated with disease progression. Hum Immunol [Internet]. 2012;73(9):946–9.

32. Zhang X, Han QY, Li JB, Ruan YY, Yan WH, Lin A. Lesion HLA-G5/-G6 isoforms expression in patients with ovarian cancer. Hum Immunol [Internet]. 2016;77(9):780–4.
33. Sun J, Chang YX, Niu CY. Evaluation of ascitic soluble human leukocyte antigen-G for distinguishing malignant ascites from benign ascites. Tumor Biol. 2017;39(11).
34. Cao M, Yie SM, Liu J, Ye SR, Xia D, Gao E. Plasma soluble HLA-G is a potential biomarker for diagnosis of colorectal, gastric, esophageal and lung cancer. Tissue Antigens. 2011;78(2):120–8.
35. Jeong S, Park S, Park BW, Park Y, Kwon OJ, Kim HS. Human leukocyte antigen-G (HLA-G) polymorphism and expression in breast cancer patients. PLoS One. 2014;9(5).
36. Pan YQ, Ruan YY, Peng JB, Han QY, Zhang X, Lin A, et al. Diagnostic significance of soluble human leukocyte antigen-G for gastric cancer. Hum Immunol [Internet]. 2016;77(4):317–24.
37. Chen HX, Lin A, Shen CJ, Zhen R, Chen BG, Zhang X, et al. Upregulation of human leukocyte antigen-G expression and its clinical significance in ductal breast cancer. Hum Immunol [Internet]. 2010;71(9):892–8.
38. Sayed D, Badr G, Maximous D, Mikhail NNH, Abu-Tarboush F, Alhazza IM. HLA-G and its relation to proliferation index in detection and monitoring breast cancer patients. Tissue Antigens. 2010;75(1):40–7.
39. Bainbridge D, Ellis S, Le Bouteiller P, Sargent I. HLA-G remains a mystery. Trends Immunol. 2001;22(10):548–52.
40. Morandi F, Pozzi S, Carlini B, Amoroso L, Pistoia V, Corrias MV. Soluble HLA-G and HLA-E Levels in Bone Marrow Plasma Samples Are Related to Disease Stage in Neuroblastoma Patients. J Immunol Res. 2016;2016.
41. Eskandari-Nasab E, Hashemi M, Hasani SS, Omrani M, Taheri M, Mashhadi MA. Association between HLA-G 3’UTR 14-bp ins/del polymorphism and susceptibility to breast cancer. Cancer Biomarkers. 2013;13(4):253–9.
42. Martelli-Palomino G, Pancotto JA, Muniz YC, Mendes-Junior CT, Castelli EC, Massaro JD, et al. Polymorphic Sites at the 3’ Untranslated Region of the HLA-G Gene Are Associated with Differential hla-g Soluble Levels in the Brazilian and French Population. PLoS One. 2013;8(10):1–10.

43. Poras I, Yaghi L, Martelli-Palomino G, Mendes CT, Muniz YCN, Cagnin NF, et al. Haplotypes of the HLA-G 3′ untranslated region respond to endogenous factors of HLA-G+ and HLA-G- cell lines differentially. PLoS One. 2017;12(1):1–18.
44. Li T, Huang H, Liao D, Ling H, Su B, Cai M. Genetic polymorphism in HLA-G 3′UTR 14-bp ins/del and risk of cancer: a meta-analysis of case–control study. Mol Genet Genomics. 2015;290(4):1235–45.
45. Hahn EC, Zambra FMB, Kamada AJ, Delongui F, Grion CMC, Reiche EMV, et al. Association of HLA-G 3′UTR polymorphisms and haplotypes with severe sepsis in a Brazilian population. Hum Immunol [Internet]. 2017;78(11–12):718–23.
46. Zambra FMB, Biolchi V, de Cerqueira CCS, Brum IS, Castelli EC, Chies JAB. Immunogenetics of prostate cancer and benign hyperplasia--the potential use of an HLA-G variant as a tag SNP for prostate cancer risk. Hla. 2016;87(2):79–88.
47. Garziera M, Catamo E, Crovella S, Montico M, Cecchin E, Lonardi S, et al. Association of the HLA-G 3′UTR polymorphisms with colorectal cancer in Italy: A first insight. Int J Immunogenet. 2016;43(1):32–9.
48. Curigliano G, Criscitiello C, Gelao L, Goldhirsch A. Molecular pathways: Human leukocyte antigen G(HLA-G). Clin Cancer Res. 2013;19(20):5564–71.
49. Siu AL. Screening for breast cancer: U.S. Preventive services task force recommendation statement. Ann Intern Med. 2016;164(4):279–96.
50. Nelson HD, Pappas M, Cantor A, Griffin J, Daeges M, Humphrey L. Harms of breast cancer screening: Systematic review to update the 2009 U.S. Preventive services task force recommendation. Ann Intern Med. 2016;164(4):256–67.
51. Miglioretti DL, Lange J, Van Den Broek JJ, Lee CI, Van Ravesteyn NT, Ritley D, et al. Radiation-induced breast cancer incidence and mortality from digital mammography screening a modeling study. Ann Intern Med. 2016;164(4):205–14.
52. Rizzo R, Fainardi E, Rouas-Freiss N, Morandi F. The Role of HLA-Class Ib Molecules in Immune-Related Diseases, Tumors, and Infections 2016. J Immunol Res. 2017;2017.
53. Carosella ED, Rouas-Freiss N, Paul P, Dausset J. HLA-G: A tolerance molecule from the major histocompatibility complex. Immunol Today. 1999;20(2):60–2.
54. Rebmann V, Regel J, Stolke D, Grosse-Wilde H. Secretion of sHLA-G molecules in malignancies. Semin Cancer Biol. 2003;13(5):371–7.

55. Kleinberg L, Flørenes VA, Skrede M, Dong HP, Nielsen S, McMaster MT, et al. Expression of HLA-G in malignant mesothelioma and clinically aggressive breast carcinoma. Virchows Arch. 2006;449(1):31–9.
56. Feng Y, Spezia M, Huang S, Yuan C, Zeng Z, Zhang L, et al. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes Dis [Internet]. 2018;5(2):77–106.
57. World Health Organization, Cancer Research UK. World cancer factsheet. World Heal Organ [Internet]. 2014;2012(2012):4.
58. Wang H, Naghavi M, Allen C, Barber RM, Carter A, Casey DC, et al. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016;388(10053):1459–544.
59. Adeloye D, Sowunmi OY, Jacobs W, David RA, Adeosun AA, Amuta AO, et al. Estimating the incidence of breast cancer in Africa: a systematic review and meta-analysis. J Glob Health [Internet]. 2018;8(1).
60. Ozsoy A, Barca N, Akdal Dolek B, Aktas H, Elverici E, Araz L, et al. The Relationship Between Breast Cancer and Risk Factors: A Single-Center Study. Eur J Breast Heal [Internet]. 2017;13(3):145–9.
61. Boice Jr. JD, Land CE, Shore RE, Norman JE, Tokunaga M. Risk of breast cancer following low-dose radiation exposure. Radiology. 1979;131(3):589–97.
62. Angahar LT. An Overview of Breast Cancer Epidemiology, Risk Factors, Pathophysiology, and Cancer Risks Reduction. MOJ Biol Med [Internet]. 2017;1(4):92–6.
63. Thorbjarnardottir T, Olafsdottir EJ, Valdimarsdottir UA, Olafsson O, Tryggvadottir L. Oral contraceptives, hormone replacement therapy and breast cancer risk: A cohort study of 16 928 women 48 years and older. Acta Oncol (Madr). 2014;53(6):752–8.
64. Byrne C, Webb PM, Jacobs TW, Peiro G, Schnitt SJ, Connolly JL, et al. Alcohol consumption and incidence of benign breast disease. Cancer EpidemiolBiomarkers Prev. 2002;11(1055–9965 (Print)):1369–74.
65. Breast C, Be C. Breast Cancer Risk and Prevention Risk Factors for Breast Cancer Breast Cancer Risk Factors You Cannot Change. Amercan Cancer Soc. 2016;

66. Ilic M, Vlajinac H, Marinkovic J. Cigarette Smoking and Breast Cancer: a Case-control Study in Serbia. Asian Pacific J Cancer Prev [Internet]. 2013;14(11):6643–7.
67. Ippoliti G, Rinaldi M, Pellegrini C, Viganò M. Incidence of cancer after immunosuppressive treatment for heart transplantation. Crit Rev Oncol Hematol. 2005;56(1 SPEC. ISS.):101–13.
68. Patterson RE, Cadmus LA, Emond JA, Pierce JP. Physical activity, diet, adiposity and female breast cancer prognosis: A review of the epidemiologic literature. Maturitas [Internet]. 2010;66(1):5–15.
69. Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G. Breast cancer. Lancet [Internet]. 2005;365(9472):1727–41.
70. Greene F, Page D, Fleming I, Fritz A. Breast [Internet]. AJCC Cancer Staging Manual. 2017. 589-636 p.
71. For O, Health P. The early detection and diagnosis of breast cancer : a literature review – a n update. Vol. 2, Health Technology Assessment. 1999.
72. Williams GH, Stoeber K. The cell cycle and cancer. J Pathol. 2012;226(2):352–64.
73. Heitz F, Morris MC, Fesquet D, Cavadore JC, Dorée M, Divita G. Interactions of cyclins with cyclin-dependent kinases: A common interactive mechanism. Biochemistry. 1997;36(16):4995–5003.
74. Lee EYHP, Muller WJ. Oncogenes and tumor suppressor genes. Cold Spring Harb Perspect Biol [Internet]. 2010;2(10):a003236.
75. Vigneron N. Human Tumor Antigens and Cancer Immunotherapy. Biomed Res Int. 2015;2015.
76. Pardoll D. Cancer and the Immune System: Basic Concepts and Targets for Intervention. Semin Oncol. 2015;42(4):523–538.
77. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: From immunosurveillance to tumor escape. Nat Immunol. 2002;3(11):991–8.
78. Calì B, Molon B, Viola A. Tuning cancer fate: the unremitting role of host immunity. Open Biol [Internet]. 2017;7(4):170006.
79. Bennett JA, Pilon VA, Macdowell RT. Evaluation of Growth and Histology of Human Tumor Xenografts Implanted under the Renal Capsule of Immunocompetent and Immunodeficient Mice Evaluation of Growth and Histology of Human Tumor Xenografts

Implanted under the Renal Capsule of Immunocompetent an. Cancer Res. 1985;45(October):4963–9.

80. Rodríguez-Perálvarez M, De La Mata M, Burroughs AK. Liver transplantation: Immunosuppression and oncology. Curr Opin Organ Transplant. 2014;19(3):253–60.
81. Gjersvik P, Helsing P, Holdaas H, Bergan S. Immunsuppressive legemidler og utvikling av hudkreft etter organtransplantasjon. Tidsskr den Nor Laegeforening. 2012;132(18):2064–8.
82. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNγ, and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410(6832):1107–11.
83. Goldsby RA, Kindt TJ, Osborne BA, Kuby J. Cancer and the Immune System. In: Immunology. 5th ed. New York, USA: W. H. Freeman; 2003.
84. Rahir G, Moser M. Tumor microenvironment and lymphocyte infiltration. Cancer Immunol Immunother. 2012;61(6):751–9.
85. Geng Y, Shao Y, He W, Hu W, Xu Y, Chen J, et al. Prognostic role of tumor-infiltrating lymphocytes in lung cancer: A meta-analysis. Cell Physiol Biochem. 2015;37(4):1560–71.
86. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci [Internet]. 2005;102(51):18538–43.
87. Mao Y, Qu Q, Zhang Y, Liu J, Chen X, Shen K. The value of tumor infiltrating lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in breast cancer: A systematic review and meta-analysis. PLoS One. 2014;9(12):1–21.
88. Xu DP, Shi WW, Zhang TT, Lv HY, Li JB, Lin A, et al. Elevation of HLA-G-expressing DC-10 cells in patients with gastric cancer. Hum Immunol [Internet]. 2016;77(9):800–4. Available from: http://dx.doi.org/10.1016/j.humimm.2016.01.003
89. Snell GD. Studies in histocompatibility. Scand J Immunol [Internet]. 1992;36(4):514–25. Available from: https://www.nobelprize.org/nobel_prizes/medicine/laureates/1980/snell-lecture.pdf
90. Naik S. the Human Hla System. J Indian Rheumatol Assoc. 2003;11:79–83.
91. Thorsby E. MHC structure and function. Transplant Proc. 1999;31(1–2):713–6.

92. Bukur J, Jasinski S, Seliger B. The role of classical and non-classical HLA class I antigens in human tumors. Semin Cancer Biol [Internet]. 2012;22(4):350–8. Available from: http://dx.doi.org/10.1016/j.semcancer.2012.03.003
93. Alegre E, Rizzo R, Bortolotti D, Fernandez-Landázuri S, Fainardi E, González A. Some basic aspects of HLA-G biology. J Immunol Res. 2014;2014.
94. Rizzo R, Trentini A, Bortolotti D, Manfrinato MC, Rotola A, Castellazzi M, et al. Matrix metalloproteinase-2 (MMP-2) generates soluble HLA-G1 by cell surface proteolytic shedding. Mol Cell Biochem. 2013;381(1–2):243–55.
95. Zidi I, Guillard C, Marcou C, Krawice-Radanne I, Sangrouber D, Rouas-Freiss N, et al. Increase in HLA-G1 proteolytic shedding by tumor cells: A regulatory pathway controlled by NF-κB inducers. Cell Mol Life Sci. 2006;63(22):2669–81.
96. König L, Kasimir-Bauer S, Hoffmann O, Bittner AK, Wagner B, Manvailer LFS, et al. The prognostic impact of soluble and vesicular HLA-G and its relationship to circulating tumor cells in neoadjuvant treated breast cancer patients. Hum Immunol. 2016;77(9):791–9.
97. LeMaoult J, Caumartin J, Daouya M, Favier B, Le Rond S, Gonzalez A, et al. Immune regulation by pretenders: Cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells. Blood. 2007;109(5):2040–8.
98. Diehl M, Münz C, Keilholz W, Stevanović S, Holmes N, Loke YW, et al. Nonclassical HLA-G molecules are classical peptide presenters. Curr Biol. 1996;6(3):305–14.
99. Boyson JE, Erskine R, Whitman MC, Chiu M, Lau JM, Koopman LA, et al. Disulfide bond-mediated dimerization of HLA-G on the cell surface. Proc Natl Acad Sci [Internet]. 2002;99(25):16180–5.
100. Rouas-Freiss N, LeMaoult J, Verine J, Tronik-Le Roux D, Culine S, Hennequin C, et al. Intratumor heterogeneity of immune checkpoints in primary renal cell cancer: Focus on HLA-G/ILT2/ILT4. Oncoimmunology [Internet]. 2017;6(9):e1342023. Available from: https://www.tandfonline.com/doi/full/10.1080/2162402X.2017.1342023
101. Svendsen SG, Hantash BM, Zhao L, Faber C, Bzorek M, Nissen MH, et al. The expression and functional activity of membrane-bound human leukocyte antigen-G1 are influenced by the 3'-untranslated region. Hum Immunol [Internet]. 2013;74(7):818–27.

102. Haghi M, Feizi MAH, Sadeghizadeh M, Lotfi AS. 14-bp Insertion / Deletion Polymorphism of the HLA-G gene in Breast Cancer among Women from North Western Iran. Asian Pacific J Cancer Prev. 2015;16:6155–8.
103. Hashemi M, Sandoughi M, Fazeli SA, Bahari G, Rezaei M, Zakeri Z. Evaluation of HLA-G 14 bp Ins/Del and +3142G>C Polymorphism with Susceptibility and Early Disease Activity in Rheumatoid Arthritis. Adv Med [Internet]. 2016;2016:1–7.
104. Alizadeh N, Mosaferi E, Farzadi L, Majidi J, Monfaredan A, Yousefi B, et al. Frequency of null allele of Human Leukocyte Antigen-G (HLA-G) locus in subjects to recurrent miscarriage. Int J Reprod Biomed (Yazd, Iran). 2016;14(7):459–64.
105. Nilsson LL, Djurisic S, Andersen AMN, Melbye M, Bjerre D, Ferrero-Miliani L, et al. Distribution of HLA-G extended haplotypes and one HLA-E polymorphism in a large-scale study of mother–child dyads with and without severe preeclampsia and eclampsia. Hla. 2016;88(4):172–86.
106. Castelli EC, Veiga-Castelli LC, Yaghi L, Moreau P, Donadi EA. Transcriptional and posttranscriptional regulations of the HLA-G gene. J Immunol Res. 2014;2014.
107. Donadi EA, Castelli EC, Arnaiz-Villena A, Roger M, Rey D, Moreau P. Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association. Cell Mol Life Sci. 2011;68(3):369–95.
108. Castelli EC, Mendes-Junior CT, Veiga-Castelli LC, Roger M, Moreau P, Donadi EA. A comprehensive study of polymorphic sites along the HLA-G gene: Implication for gene regulation and evolution. Mol Biol Evol. 2011;28(11):3069–86.
109. Ferreira LMR, Meissner TB, Mikkelsen TS, Mallard W, O'Donnell CW, Tilburgs T, et al. A distant trophoblast-specific enhancer controls HLA-G expression at the maternal–fetal interface. Proc Natl Acad Sci [Internet]. 2016;113(19):5364–9.
110. Garziera M, Scarabel L, Toffoli G. Hypoxic Modulation of HLA-G Expression through the Metabolic Sensor HIF-1 in Human Cancer Cells. J Immunol Res. 2017;2017.
111. Macklin PS, McAuliffe J, Pugh CW, Yamamoto A. Hypoxia and HIF pathway in cancer and the placenta. Placenta [Internet]. 2017;56:8–13.
112. Brindle P, Nakajima T, Montminy M. Multiple protein kinase A-regulated events are required for transcriptional induction by cAMP. Proc Natl Acad Sci [Internet]. 1995;92(23):10521–5.

113. Amiot L, Onno M, Drenou B, Monvoisin C, Fauchet R. HLA-G class I gene expression in normal and malignant hematopoietic cells. Hum Immunol [Internet]. 1998;59(8):524–8.
114. Onno M, Guillaudeux T, Amiot L, Renard I, Drenou B, Hirel B, et al. The HLA-G gene is expressed at a low mRNA level in different human cells and tissues. Hum Immunol [Internet]. 1994;41(1):79–86.
115. Manaster I, Goldman-Wohl D, Greenfield C, Nachmani D, Tsukerman P, Hamani Y, et al. MiRNA-mediated control of HLA-G expression and function. PLoS One. 2012;7(3):1–9.
116. Zhu X ming, Han T, Wang X hong, Li Y hong, Yang H guang, Luo Y ning, et al. Overexpression of miR-152 leads to reduced expression of human leukocyte antigen-G and increased natural killer cell mediated cytolysis in JEG-3 cells. Am J Obstet Gynecol [Internet]. 2010;202(6):592.e1-592.e7.
117. Seliger B. Role of microRNAs on HLA-G expression in human tumors. Hum Immunol [Internet]. 2016;77(9):760–3.
118. Chen Q, Luo G, Zhang X. MiR-148a modulates HLA-G expression and influences tumor apoptosis in esophageal squamous cell carcinoma. Exp Ther Med. 2017;14(5):4448–52.
119. Mori A, Nishi H, Sasaki T, Nagamitsu Y, Kawaguchi R, Okamoto A, et al. HLA-G expression is regulated by miR-365 in trophoblasts under hypoxic conditions. Placenta [Internet]. 2016;45:37–41.
120. Holling TM, Bergevoet MWT, Wierda RJ, Van Eggermond MCJA, Van Den Elsen PJ. Genetic and Epigenetic Control of the Major Histocompatibility Complex Class Ib Gene HLA-G in Trophoblast Cell Lines. Ann N Y Acad Sci [Internet]. 2009;1173(1):538–44.
121. Swets M, Seneby L, Boot A, van Wezel T, Gelderblom H, van de Velde CJH, et al. Promoter methylation and mRNA expression of HLA-G in relation to HLA-G protein expression in colorectal cancer. Hum Immunol [Internet]. 2016;77(9):764–72.
122. Poddar S, Kesharwani D, Datta M. Interplay between the miRNome and the epigenetic machinery: Implications in health and disease. J Cell Physiol. 2017;232(11):2938–45.
123. Bielska M, Bojo M, Klimkiewicz-Wojciechowska G, Jesionek-Kupnicka D, Borowiec M, Kalinka-Warzocha E, et al. Human Leukocyte Antigen-G Polymorphisms Influence the Clinical Outcome in Diffuse Large B-Cell Lymphoma. GENES, Chromosom CANCER 54185–193. 2015;54:185–193.

124. Hachiya Y, Kawasaki A, Oka S, Kondo Y, Ito S, Matsumoto I, et al. Association of HLA-G 3' Untranslated region polymorphisms with systemic lupus erythematosus in a Japanese population: A case-control association study. PLoS One. 2016;11(6):1–14.

125. Ober C, Billstrand C, Kuldanek S, Tan Z. The miscarriage-associated HLA-G -725G allele influences transcription rates in JEG-3 cells. Hum Reprod. 2006;21(7):1743–8.

126. Alves BM, Prellwitz IM, Siqueira JD, Meyrelles ÂR, Bergmann A, Seuánez HN, et al. Infection , Genetics and Evolution The effect of human leukocyte antigen G alleles on human papillomavirus infection and persistence in a cohort of HIV-positive pregnant women from Brazil. Infect Genet Evol [Internet]. 2015;34:339–43.

127. Zidi I, Dziri O, Zidi N, Sebai R, Boujelebene N, Ben Hassine A, et al. Association of HLA-G +3142 C>G polymorphism and breast cancer in Tunisian population. Immunol Res. 2016;64(4):961–8.

128. Biolchi V, Cerqueira CCS De, Brum IS, Castelli EC, Chies JAB. Immunogenetics of prostate cancer and benign hyperplasia – the potential use of an HLA-G variant as a tag SNP for prostate cancer risk. 2016;(5):79–88.

129. Hashemi M, Sandoughi M, Fazeli SA, Bahari G, Rezaei M, Zakeri Z. Evaluation of HLA-G 14 bp Ins/Del and +3142G>C Polymorphism with Susceptibility and Early Disease Activity in Rheumatoid Arthritis. Adv Med [Internet]. 2016;2016:1–7.

130. Sabbagh A, Luisi P, Castelli EC, Gineau L, Courtin D, Milet J, et al. Worldwide genetic variation at the 3′ untranslated region of the HLA-G gene: Balancing selection influencing genetic diversity. Genes Immun [Internet]. 2014;15(2):95–106.

131. Khorrami S, Rahimi R, Mohammadpour H, Bahrami S, Yari F, Poustchi H, et al. Association of HLA-G*01:01:02:01/G*01:04:01 polymorphism with gastric adenocarcinoma. Hum Immunol [Internet]. 2016;77(2):153–7.

132. Ben Amor A, Beauchemin K, Faucher M-C, Hamzaoui A, Hamzaoui K, Roger M. Human Leukocyte Antigen G Polymorphism and Expression Are Associated with an Increased Risk of Non-Small-Cell Lung Cancer and Advanced Disease Stage. PLoS One [Internet]. 2016;11(8):e0161210.

133. Kowal A, Wiśniewski A, Kuśnierczyk P, Jankowska R. Human leukocyte antigen (HLA)-G gene polymorphism in patients with non-small cell lung cancer. Thorac Cancer. 2015;6(5):613–9.

134. Verbruggen LA, Rebmann V, Demanet C, De Cock S, Grosse-Wilde H. Soluble HLA-G in Rheumatoid Arthritis. Hum Immunol. 2006;67(8):561–7.

135. Lozano JM, González R, Kindelán JM, Rouas-Freiss N, Caballos R, Dausset J, et al. Monocytes and T lymphocytes in HIV-1-positive patients express HLA-G molecule. Aids. 2002;16(3):347–51.

136. Yan W, Lin A, Chen B, Chen S. Induction of Both Membrane-Bound and Soluble HLA-G Expression in Active Human Cytomegalovirus Infection. J Infect Dis [Internet]. 2009;200(5):820–6.

137. Paul P, Rouas-Freiss N, Khalil-Daher I, Moreau P, Riteau B, Le Gal FA, et al. HLA-G expression in melanoma: A way for tumor cells to escape from immunosurveillance. Proc Natl Acad Sci [Internet]. 1998;95(8):4510–5.

138. Nazari N, Farjadian S. Natural Killer Cell Cytotoxicity Against SKOV3 after HLA-G Downregulation by shRNA. Iran J Immunol. 2016;13(3):178–85.

139. Veluchamy JP, Heeren AM, Spanholtz J, van Eendenburg JDH, Heideman DAM, Kenter GG, et al. High-efficiency lysis of cervical cancer by allogeneic NK cells derived from umbilical cord progenitors is independent of HLA status. Cancer Immunol Immunother. 2017;66(1):51–61.

140. Da Silva IL, Montero-Montero L, Martín-Villar E, Martin-Pérez J, Sainz B, Renart J, et al. Reduced expression of the murine HLA-G homolog Qa-2 is associated with malignancy, epithelial-mesenchymal transition and stemness in breast cancer cells. Sci Rep. 2017;7(1):1–11.

141. da Silva IL, Veloso ES, Gonçalves INN, Braga AD, Lopes MTP, Cassali GD, et al. Qa-2 expression levels is related with tumor-infiltrating lymphocytes profile during solid Ehrlich tumor development. Biomed Pharmacother [Internet]. 2017;92:750–6.

142. Contini P, Ghio M, Poggi A, Filaci G, Indiveri F, Ferrone S, et al. Soluble HLA-A,-B,-C and -G molecules induce apoptosis in T and NK CD8+ cells and inhibit cytotoxic T cell activity through CD8 ligation. Eur J Immunol. 2003;33(1):125–34.

143. Gimenes F, Teixeira JJV, de Abreu ALP, Souza RP, Pereira MW, da Silva VRS, et al. Human leukocyte antigen (HLA)-G and cervical cancer immunoediting: A candidate molecule for therapeutic intervention and prognostic biomarker? Biochim Biophys Acta - Rev Cancer. 2014;1846(2):576–89.

144. Melsted WN, Matzen SH, Andersen MH, Hviid TVF. The choriocarcinoma cell line JEG-3 upregulates regulatory T cell phenotypes and modulates pro-inflammatory cytokines through HLA-G. Cell Immunol [Internet]. 2017;(November):0–1.
145. Ostapchuk YO, Cetin EA, Perfilyeva Y V., Yilmaz A, Skiba YA, Chirkin AP, et al. Peripheral blood NK cells expressing HLA-G, IL-10 and TGF-β in healthy donors and breast cancer patients. Cell Immunol [Internet]. 2015;298(1–2):37–46.
146. Gonçalves AS, Mosconi C, Jaeger F, Wastowski IJ, Aguiar MCF, Silva TA, et al. Overexpression of immunomodulatory mediators in oral precancerous lesions. Hum Immunol [Internet]. 2017;78(11–12):752–7.
147. Swets M, König MH, Zaalberg A, Dekker-Ensink NG, Gelderblom H, van de Velde CJH, et al. HLA-G and classical HLA class I expression in primary colorectal cancer and associated liver metastases. Hum Immunol [Internet]. 2016;77(9):773–9.
148. Ugurel S, Rebmann V, Ferrone S, Tilgen W, Grosse-Wilde H, Reinhold U. Soluble human leukocyte antigen-G serum level is elevated in melanoma patients and is further increased by interferon-alpha immunotherapy. Cancer [Internet]. 2001;92(2):369–76.
149. Tronik-Le Roux D, Renard J, Vérine J, Renault V, Tubacher E, LeMaoult J, et al. Novel landscape of HLA-G isoforms expressed in clear cell renal cell carcinoma patients. Mol Oncol. 2017;11(11):1561–78.
150. Yaghi L, Poras I, Simoes RT, Donadi EA, Tost J, Daunay A, et al. Hypoxia inducible factor-1 mediates the expression of the immune checkpoint HLA-G in glioma cells through hypoxia response element located in exon 2. Oncotarget [Internet]. 2016;7(39):63690–707.
151. Yang Y, Geraghty DE, Hunt JS. Cytokine regulation of HLA-G expression in human trophoblast cell lines. J Reprod Immunol. 1995;29(3):179–95.
152. de Figueiredo-Feitosa NL, Martelli Palomino G, Cilião Alves DC, Mendes Junior CT, Donadi EA, Maciel LMZ. HLA-G 3′ untranslated region polymorphic sites associated with increased HLA-G production are more frequent in patients exhibiting differentiated thyroid tumours. Clin Endocrinol (Oxf). 2017;86(4):597–605.
153. Mosconi C, Arantes DAC, Gonçalves AS, Alencar R de CG, Oliveira JC, Silva TA, et al. Immunohistochemical investigations on the expression of programmed cell death ligand 1, human leukocyte antigens G and E, and granzyme B in intraoral mucoepidermoid carcinoma. Arch Oral Biol [Internet]. 2017;83(February):55–62.

154. Davies B, Hiby S, Gardner L, Loke YW, King A. HLA-G expression by tumors. Am J Reprod Immunol. 2001;45(2):103–7.
155. Pangault C, Amiot L, Caulet-Maugendre S, Brasseur F, Burtin F, Guilloux V, et al. HLA-G protein expression is not induced during malignant transformation. Tissue Antigens. 1999;53(4 I):335–46.
156. Real LM, Cabrera T, Collado A, Jimenez P, Garcia A, Ruiz-Cabello F, et al. Expression of HLA G in human tumors is not a frequent event. Int J Cancer. 1999;81(4):512–8.
157. Frumento G, Franchello S, Palmisano GL, Nicotra MR, Giacomini P, Loke YW, et al. Melanomas and melanoma cell lines do not express HLA-G, and the expression cannot be induced by gammaIFN treatment. Tissue Antigens [Internet]. 2000;56(1):30–7.
158. Gao M, Gao L, Yang G, Tao Y, Hou J, Xu H, et al. Myeloma cells resistance to NK cell lysis mainly involves an HLA class I-dependent mechanism. Acta Biochim Biophys Sin (Shanghai). 2014;46(7):597–604.
159. Heidari MH, Movafagh A, Abdollahifar M-A, Abdi S, Barez MM, Azimi H, et al. Erratum to: Evaluation of sHLA-G levels in serum of patients with prostate cancer identify as a potential of tumor marker [Anat Cell Biol., 50, 1, (2017) (69-72)] DOI: 10.5115/acb.2017.50.1.69. Anat Cell Biol [Internet]. 2017;50(2):162.
160. Singer G, Rebmann V, Chen Y-C, Liu H-T, Ali SZ, Reinsberg J, et al. HLA-G Is a Potential Tumor Marker in Malignant Ascites. Clin Cancer Res [Internet]. 2003;9(12):4460–4.
161. Motawi TMK, Zakhary NI, Salman TM, Tadros S a. Serum human leukocyte antigen-G and soluble interleukin 2 receptor levels in acute lymphoblastic leukemic pediatric patients. Asian Pac J Cancer Prev [Internet]. 2012;13(11):5399–403.
162. Gan LH, Huang LF, Zhang X, Lin A, Xu DP, Wang Q, et al. Tumor-specific upregulation of human leukocyte antigen-G expression in bladder transitional cell carcinoma. Hum Immunol [Internet]. 2010;71(9):899–904.
163. Kester ADM, Buntinx F. Meta-analysis of ROC curves. Med Decis Mak. 2000;20(4):430–9.
164. Kumar R, Indrayan A. Receiver operating characteristic (ROC) curve for medical researchers. Indian Pediatr [Internet]. 2011;48(4):277–87.

165. Kirana C, Ruszkiewicz A, Stubbs RS, Hardingham JE, Hewett PJ, Maddern GJ, et al. Soluble HLA-G is a differential prognostic marker in sequential colorectal cancer disease stages. Int J Cancer. 2017;140(11):2577–86.
166. Rutten MJ, Dijk F, Savci-Heijink CD, Buist MR, Kenter GG, Vijver MJV De, et al. HLA-G expression is an independent predictor for improved survival in high grade ovarian carcinomas. J Immunol Res. 2014;2014.
167. Naji A, Menier C, Maki G, Carosella ED, Rouas-Freiss N. Neoplastic B-cell growth is impaired by HLA-G/ILT2 interaction. Leukemia. 2012;26(8):1889–92.
168. Yong P, Kim SJ, Lee SJ, Kim BS. Serum level of soluble human leukocyte antigen-G molecules in non-Hodgkin lymphoma: Does it have a prognostic value? Leuk Lymphoma. 2008;49(8):1623–6.
169. Ferns DM, Heeren AM, Samuels S, Bleeker MCG, de Gruijl TD, Kenter GG, et al. Classical and non-classical HLA class I aberrations in primary cervical squamous- and adenocarcinomas and paired lymph node metastases. J Immunother Cancer [Internet]. 2016;4(1):1–11.
170. Agaugué S, Carosella ED, Rouas-Freiss N. Role of HLA-G in tumor escape through expansion of myeloid-derived suppressor cells and cytokinic balance in favor of Th2 versus Th1/Th17. Blood. 2011;117(26):7021–31.
171. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human Leukocyte Antigen-G5 Secretion by Human Mesenchymal Stem Cells Is Required to Suppress T Lymphocyte and Natural Killer Function and to Induce $CD4^{+}$ $CD25^{high}$ $FOXP3^{+}$ Regulatory T Cells. Stem Cells [Internet]. 2008;26(1):212–22.
172. Zeng XC, Zhang T, Huang DH, Wang GY, Chen W, Li H, et al. RNA interfering targeting human leukocyte antigen-G enhanced immune surveillance mediated by the natural killer cells on hepatocellular carcinoma. Ann Clin Lab Sci. 2013;43(2):135–44.
173. Castelli EC, Moreau P, Chiromatzo AO e, Mendes-Junior CT, Veiga-Castelli LC, Yaghi L, et al. In silico analysis of microRNAS targeting the HLA-G 3′ untranslated region alleles and haplotypes. Hum Immunol [Internet]. 2009;70(12):1020–5.
174. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the Areas under Two or More Correlated Receiver Operating Characteristic Curves: A Nonparametric Approach. Biometrics [Internet]. 1988;44(3):837.

175. Fruci D, Benevolo M, Cifaldi L, Lorenzi S, Lo Monaco E, Tremante E, et al. Major histocompatibility complex class i and tumour immuno-evasion: How to fool T cells and natural killer cells at one time. Curr Oncol. 2012;19(1):39–41.
176. Baudhuin J, Lesport E, Sousa S, Migraine J, Vigneron J, Lemaoult J, et al. HLA-G inhibition of NK-cell cytolytic function is uncoupled from tumor cell lipid raft reorganization. Eur J Immunol. 2012;42(3):700–9.
177. Sugita S, Makabe K, Iwasaki Y, Fujii S, Takahashi M. Natural Killer Cell Inhibition by HLA-E Molecules on Induced Pluripotent Stem Cell–Derived Retinal Pigment Epithelial Cells. Investig Opthalmology Vis Sci [Internet]. 2018;59(5):1719.
178. Petrie EJ, Clements CS, Lin J, Sullivan LC, Johnson D, Huyton T, et al. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. J Exp Med [Internet]. 2008;205(3):725–35.
179. Akin M, Aral LA, Yavuz A, Karabacak H, Dıkmen K, Bostanci H. Plasma human leukocyte antigen-G (HLA-G) in patients with thyroid cancer. Turkish J Med Sci. 2017;47(4):1263–6.
180. Yie SM, Xiao R, Librach CL. Progesterone regulates HLA-G gene expression through a novel progesterone response element. Hum Reprod. 2006;21(10):2538–44.
181. Mitsdoerffer M, Schreiner B, Kieseier BC, Neuhaus O, Dichgans J, Hartung HP, et al. Monocyte-derived HLA-G acts as a strong inhibitor of autologous CD4 T cell activation and is upregulated by interferon-β in vitro and in vivo: Rationale for the therapy of multiple sclerosis. J Neuroimmunol. 2005;159(1–2):155–64.
182. Sebti Y, Le Friec G, Pangault C, Gros F, Drénou B, Guilloux V, et al. Soluble HLA-G Molecules are Increased in Lymphoproliferative Disorders. Hum Immunol. 2003;64(11):1093–101.
183. Gros F, Sebti Y, de Guibert S, Branger B, Bernard M, Fauchet R, et al. Soluble HLA-G molecules increase during acute leukemia, especially in subtypes affecting monocytic and lymphoid lineages. Neoplasia [Internet]. 2006;8(3):223–30.
184. Rudstein-Svetlicky N, Loewenthal R, Horejsi V, Gazit E. HLA-G levels in serum and plasma. Tissue Antigens. 2007;69(SUPPL. 1):140–2.
185. Yie SM, Li LH, Li YM, Librach C. HLA-G protein concentrations in maternal serum and placental tissue are decreased in preeclampsia. Am J Obstet Gynecol. 2004;191(2):525–9.
186. Real LM, Cabrera T, Canton J, Oliva R, Ruiz-Cabello F, Garrido F. Looking for HLA-G expression in human tumours. J Reprod Immunol. 1999;43(2):263–73.

7. APPENDICES

- Written Consent form (English)
- Written Consent form (Swahili)
- Questionnaire (English)
- Questionnaire (Swahili)
- Data Collection sheet

Appendix i

INFORMED CONSENT

INTRODUCTION

Greetings. My name is Ismael C. Adolf, a Masters student in Biochemistry at Muhimbili University of Health and Allied Sciences (MUHAS). I am conducting a research on "*Clinical implication of soluble HLA-G and immunogenetic influence of HLA-G 3142G/C polymorphism in cervical and breast cancer development among patients attending ORCI in Tanzania*". I would like to invite you to take part in this study. Firstly, I will talk to you about the study and then I will ask you to give me your blood sample.

PURPOSE OF THE STUDY

The purpose of this study is to investigate the utility of soluble HLA-G molecule in diagnosis and determination of progression of breast and cervical cancer. Apart from that, genetic configuration of HLA-G molecule will be investigated as a determinant for breast and cervical cancer susceptibility. The study will help in improving the existing diagnostic and prognostic methods. The findings will also further the understanding of the genetic markers for breast and cervical cancer susceptibility, which can be used for screening people at risk for these diseases. You are requested to be part of this study because you meet the requirements required for this study.

WHAT PARTICIPATION INVOLVES

If you agree to participate in this study, you will be required to provide some information such as your particulars and other information concerning the study. Thereafter, you will be required to give blood sample for the purpose of this study.

RISK

No any risk expected happening to you because of joining this study.

BENEFITS

By your willingness to provide sample for this study, you will have contributed in search for improved way of determining the progress of cancer and its diagnosis, and further understanding of the genetic marker in question for breast and cervical cancer. All these three are important in screening and early detection, and early treatment of breast and cervical cancer.

CONFIDENTIALITY

The information collected from you and results that we obtain from this study will be kept strictly confidential. Only people that are involved in this study will be able to access the information.

RIGHTS TO WITHDRAW AND ALTERNATIVES

Taking part in this study is entirely one`s choice. There is no harm if you decide to no longer participate in the study. You are free to stop participating in this study at any time even though you have already given your consent.

WHOM TO CONTACT

If you ever have questions about this study, you should contact the Principal Investigator, Ismael C. Adolf, Department of Biochemistry, Muhimbili University of Health and Allied Sciences (MUHAS), P.O. Box 65001, Dar es Salaam (Mobile number: 0754847871, Email: chatitadolf@gmail.com). Should you have a question about your rights as a participant, you may contact: Dr. Ted Mselle, My supervisor, Department of Biochemistry, P. O. Box 65001, MUHAS.Dr. Joyce Masalu, Director, Research and Publications, P. O. Box 65001, Dar es Salaam. E-mail: drp@muhas.ac.tz, Phone: 2152489

PARTICIPANT'S DECLARATION:

I had an opportunity to discuss the study with the investigator and I am satisfied that I understand what the study involves.

I agree to participate in this study:

Patients/Parent/Guardian`s

Signature or thumb print…………………………Date………………………………

Parent/Guardian`s Name……………………………………………………….

Witness

Signature (If caretaker cannot read) ………………… Date………………………….

Witness` Name: ……………………………………………………………………….

RESEARCH OFFICER'S DECLARATION:

I certify that the above was explained verbally to the patient/parent/guardian, and that she understands the nature and the purpose of the study and consents to the participation in the study of the above patient.

I have given her the opportunity to ask questions which have been answered satisfactory.

Research Officer

Signature………………………………… Date……………………………………………

Research Officer Name: ……………………………………………………………….

Appendix ii

Kiambatanisho ii

UTANGULIZI

Salaam ndugu. Jina langu ni Ismael C. Adolf, ni mwanafunzi wa shahada ya uzamili katika masomo ya kemia hai itolewayo na chuo kikuu cha afya na sayasi shirikishi cha Muhimbili (MUHAS). Ninafanya utafiti wa namna molekyuli iitwayo "HLA-G" inavyohusika katika ukuaji wa saratani ya kizazi na matiti, na namna utofauti wa sehemu ya kinasaba inayoratibu utengenezwaji wake (HLA-G 3142G/C polymorphism) inavyoathiri utengenezwaji wake katika wagonjwa wa taasisi ya saratani ya osheni rodi (ORCI). Ningependa kukualika uwe sehemu ya utafiti huu. Kwanza nitaongea na wewe kuhusu utafiti huu halafu nitakuomba utoe kiasi kidogo cha damu kwa ajili ya utafiti.

LENGO LA UTAFITI

Lengo la utafiti huu ni kutabaisha uwezekano wa "HLA-G" iliyoko kwenye damu kutumika katika uchunguzi na ukuaji wa saratani ya kizazi na matiti. Pia, asili ya sehemu ya vinasaba inayotengeneza hii molekyuli itachunguzwa kama kiashiria cha urahisi wa kupata saratani ya kizazi na matiti. Utafiti huu utasaidia katika kuboresha huduma za upimaji na kuangalia ukuaji wa saratani ya kizazi na matiti. Utafiti huu pia utaongeza ufahamu kuhusu viashiria vya vinasaba vinavyoashiria wepesi wa kupata saratani hizi, hivyo kutumika katika "kuskrini" watu waliopo kwenye hatari ya kupata magonjwa haya. Unaombwa kuwa sehemu ya utafiti huu kwani unakidhi vigezo vinavyohitajika kwa ajili ya utafiti huu.

KINACHOHUSIKA KATIKA KUSHIRIKI

Kama ukikubali kushiriki katika utafiti huu, utatakiwa kutoa taarifa zako binafsi na nyingine zinazohusu utafiti huu. Baada ya hapo utatakiwa kutoa sampuli ya damu yako kwa ajili ya utafiti.

HATARI

Hakuna hatari yoyote inayotazamiwa kutokea kwako kwa kukubali shiriki katika utafiti huu.

FAIDA

Kwa utayari wako wa kutoa sampuli kwa ajili ya utafiti huu utakuwa umechangia katika harakati za kuboresha namna ya kupima saratani na ukuaji wake kwa kutumia damu, na kujua zaidi kuhusu viashiria vya kijenetiki vya urahisi wa kupata saratani ya kizazi na matiti. Vitu vyote hivi ni muhimu katika "kuskrini" na kugundua saratani ikiwa katika hatua za mwanzo na kupewa matibabu mapema.

USIRI

Taarifa zitakazopatikana kutoka kwako na matokeo yake vitatunzwa kwa siri kubwa. Ni wale watu pekee ambao wanahusika moja kwa moja katika utafiti huu ambao wataweza kuzipata taarifa hizi.

HAKI YA KUJITOA KWENYE UTAFITI HUU NA MBADALA

Kuwa sehemu ya utafiti huu ni maamuzi ya mtu husika. Hamna madhara kama ukiamua kutoendelea kushiriki katika utafiti huu. Uko huru kusitisha ushiriki wako hata kama ulikuwa umeshakubali mwanzoni.

MAWASILIANO

Kama una swali lolote kuhusu utafiti huu, unaweza wasiliana na mtafiti mkuu, Ismael C. Adolf, Idara ya kemia hai, chuo kikuu cha afya na sayansi shirikishi cha Muhimbili (MUHAS), S.L.P 65001, Dar es Salaam (Simu ya mkononi: 0754847871, Barua pepe: chatitadolf@gmail.com). Kama una swali lolote kuhusiana na haki yako yama mshiriki katika tafiti hii unaweza kuwasiliana na:

Msimamizi wangu, Dk. Teddy Msele, Idara ya Kemia hai, S.L.P 65001, MUHAS.

Dk Joyce Masalu, Mkurugenzi, Kurugenzi ya Utafiti na Machapisho, S. L. P 65001, Dar es Salaam. Barua pepe: drp@muhas.ac.tz, Simu: 2152489

TAMKO LA MSHIRIKI:

Nilikuwa na fursa ya kujadili na mtafiti kuhusu tafiti yake na nimeridhika na kujua kinachohusika katika tafiti hii.

Nimekubali kushiriki katika utafiti huu.

Sahihi ya au dole gumba la mhusika/mzazi/mlezi……………................tarehe...........................

Jina la mhusika/mzazi/mlezi…………………………………………………………………….

Sahihi ya shahidi (kama mhusika hawezi kusoma)…………………………tarehe………………..

Jina la shahidi……………………………………………………………………………….

TAMKO LA MTAFITI:

Nathibitisha kwamba yote yaliyoandikwa hapo juu yameelezwa kwa mdomo kwa mhusika/mzazi/mlezi na ameelewa kuhusu utafiti huu na malengo yake na kakubali kushiriki katika utafiti huu.

Nimempa nafasi ya kuuliza maswali na nimempa majibu ya kuridhisha

Mtafiti

Sahihi……………………………………. tarehe…………………………………………………

Jina la mtafiti……………………………………………………………………………….

Appendix iii

QUESTIONNAIRE

Proforma for breast and cervical cancer study: "Clinical implication of soluble HLA-G and immunogenetic influence of HLA-G 3142G/C polymorphism in cervical and breast cancer development among patients attending ORCI in Tanzania".

A: DEMOGRAPHIC HISTORY:

•Hospital ID number (HID) ____

•Name ____

•Age (years) ____

•Place of birth (District) ____

•Tribe ____

•Weight: ______ (kg) Height: _____ m____ cm

•Tribe of mother____

•Tribe of Father____

•Respondent (Self/Parent/Guardian/Other) - ______________________________

B: RESIDENCE INFORMATION

ADDRESS:

____

______ Region ___________________________________P.O. Box_________________

WORK Phone Number: ____

CELL Phone Number: ____

EMAIL ADDRESS: ____

C: RISK FACTOR DETERMINATION (Please check all spaces that apply to you.)

☐ Sedentary lifestyle ☐ Currently smoking

☐ Quit smoking ☐ Lifetime non-smoker

☐ Regular exerciser How much?

☐ Relatives withcancer/died of cancer ☐ Immediate ☐ Paternal ☐ Maternal

D: MEDICAL HISTORY

Have you had any operations? Yes ☐ No ☐

Type of operation Was it for cancer? date Hospital

Have you or anyone in your family ever suffered from a blood clot?

No ☐ yes ☐

Have you had any cancer screening such as mammography or colonoscopy? Yes No

Type of screening how often last date performed Hospital

E: REPRODUCTION RELATED INFORMATION

At what age was your first menstrual period? Age:_______

If you have children how old were you when your first child was born? Age: _____

Do/did you breast feed your children?

Yes ☐ No ☐ Not applicable ☐

If yes for how long in total? ___________

Do/did you use the oral contraceptive pill? Please tick the box that applies

No, I have never used the oral contraceptive pill ☐

Yes, I currently use the oral contraceptive pill. ☐

Yes, I have used the oral contraceptive pill in the past, but do not use it at present. ☐

If yes, for how many years have you used the pill?__________________

Do/did you use HRT? Please tick the option that applies to you:

No, I have never used HRT ☐

☐ Yes, I currently use HRT

☐ Yes, I have used HRT in the past, but do not use it at present.

If yes, for how long did you/have you taken HRT? ____________________

Which type of HRT were/are you taking: oestrogen based/combined oestrogen-progesterone

Are you in the menopause? Please tick the option that applies to you:

☐ No, I have not been through the menopause

☐ Yes, I am in/have been through the menopause

If yes, at what age did you begin the menopause? _____________

Additional information: Please tell us anything further you think is important here or use this space to mention any specific concerns or questions you have.

CLINICAL INVESTIGATOR: ________________________________

Signature____

PARTICIPANT: ____

Signature____

Appendix iv

Kiambatanisho iv

DODOSO

Profoma ya utafiti wa kansa ya matiti na kizazi: “Uhusikaji wa kikliniki wa HLA-G na ushawishi wa jenetikia ya kiimyunolojia ya “+3142 G/C” katika uanzishwaji na ukuaji wa saratani ya matiti na kizizi kwa wagonjwa wanaokuja hospitali ya saratani ya osheni rodi (ORCI).

A: HISTORIA SIFA

- Nambari ya utambulisho wa hospitali ____
- Jina/Jina la ukoo ____
- Jinsia (Me/Ke) Kike Kiume
- Umri (miaka- miezi) ____
- Mahali pa kuzaliwa (wilaya)____
- Kabila ____
- Uzito: _______ (kg) Kimo: _____ m _____cm
- Kabila la mama____
- Kabila la baba____
- Mshiriki (Mwenyewe, mzazi, mlezi)____

B: TAARIFA ZA MAKAZI

ANUANI:

____

__________ **Mkoa** ____________________________________**S.L.P** _____________________

Nambari ya simu ya kazini____

Nambari ya simu ya mkononi____

Barua pepe____

C: KUANGALIA MAMBO HATARISHI (Tafadhali angalia nafasi zote zinazokuhusu.)

□ Aina ya maisha yasiyo na mazoezi □ Unavuta sigara kwa sasa

□ Umeacha kuvuta sigara □ Katika maisha yako hujawahi kuvuta sigara

□ Unafanya mazoezi wa mara kwa mara, Kwa kiasi gani?

□ Una ndugu mwenye/aliyekufa na saratani? □ Ndugu wa karibu □ Upande wa baba □Upande waMama

D: HISTORIA YA MATIBABU

Ulishawahi fanyiwa upasuaji? NDIYO □ HAPANA □

Aina ya upasuaji__________ Ulikuwa kwa ajili ya saratani?_______________

Tarehe_____________ Hospitali_________________

Ulishawahi au mmoja wa wanafamilia alishawahi kuwa na tatizo la kuganda kwa damu?

Hapana □ Ndiyo □ if yes, who was it?

Ulishawahi kwenda fanyiwa uchunguzi wa saratani kama vile ya matiti au ya utumbo kwa kutumia mionzi? Ndiyo______ Hapana________

Aina ya uchunguzi _______________ Mara ngapi____________

Mara ya mwisho kufanyiwa___________________________ Hospitali_______________

E: TAARIFA ZINAZOHUSIANA NA UZAZI

Ulikuwa na umri gani ulipopata hedhi ya kwanza? Umri:_______

Kama una mtoto, ulikuwa na umri gani ulipopata mtoto wa kweanza? Umri: _____

Una/uliwanyonyesha maziwa yako watoto wako?

Ndiyo ☐ hapana ☐ i husika ☐

Kama ndiyo, kwa muda gani kwa ujumla? ___________

Unatumia vidonge vya uzazi wa mpango? Weka vema panapohusika

☐ Hapana, sijawahi tumia vidonge vya uzazi wa mpango

☐ Ndiyo, kwa sasa natumia vidonge vya uzazi wa mpango.

☐ Ndiyo, nilitumia vidonge vya uzazi wa mpango zamani, lakini situmii kwa sasa

Kama ndiyo, kwa muda gani umekuwa ukitumia vidonge hivyo?___________

Unatumia/ulishawahi tumia homoni mbadala (HRT)? Tafadhali weka vema panapohusika:

☐ Hapana, kamwe sijawahi tumia

☐ Ndiyo, kwa sasa natumia

☐ Ndiyo, nilitumia zamani, lakini siyo kwa sasa.

Kama ndiyo, kwa muda gani umekuwa ukitumia homoni hizo? __________

Ni aina gani ya homoni (ulikuwa) unatumia: estrojeni /mchanganyiko wa

Estrojeni na progesterone

Haupati hedhi tena? Tafadhali, weka vema panapohusika:

☐ Hapana, siawahi acha pata hedhi

☐ Ndiyo, (nimekuwa) sipati hedhi

Kama ndiyo, ulikuwa na umri gani ulipoanza kutopata hedhi? _____________

Taarifa zaidi: Tafadhali, twambie kitu chochote unachodhani ni muhimu au tumia nafasi hii chini kutaja dukuduku lako au swali ulilonalo.

Mtafiti: ___________________________________

Sahihi____

Mhusika: ____

Sahihi____

Appendix iv

DATA COLLECTION:

1- Anthropometric Measurements

Height	
Weight	
BMI	
Blood pressure	

2- Histopathological parameters

Tumor stage	
Metastatic status	
Tumor grade	
Mastectomy status	
ER expression status	
PR expression status	
HER2 expression status	

3- Blood collected (10 ml EDTA tube) for:

Genotyping rs1063320	
Immunoassaying HLA-G	