Epstein Barr Virus (EBV)-Associated Cancer biomarkers for diagnosis, prognosis and therapy: Narrative Review

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Abstract

Oncoviruses are associated with several malignancies. In EBV-associated malignancies initiation of oncogenesis associated with immune evasion, abnormal regulation of cell cycle, and targeting several host signalling pathways. Up to 70% of human cancers are associated with viruses. Clinical biomarkers are biological markers that can be used for diagnostic, prognostic, and evaluation of therapeutic responses. EBV also referred to as HHV4 is ubiquitous virus that belong to y-herpesviruses family and is associated with a number of cancers. About 318 000 new cases and 209 000 of deaths were associated with EBV-associated malignancies in 2020. Viral load, immunological profiling, EBV oncoproteins, ctDNA are potential biomarkers for EBV-associated malignancies. Multiomics approach is one of the strategies that can be utilized in identification of biomarkers in EBV- and viral-associated malignancies. The aim of this review is to provide an update on EBV-associated malignancies and the predictive biomarkers that can be utilized for diagnostic, prognostic and elucidation of therapeutic responses.

Keywords: Oncoviruses, EBV, biomarkers, diagnosis, prognosis, therapy

Human Viruses & Cancer

Oncoviruses are viruses that cause different cancers. To enable them survive, these viruses have evolved strategies that have direct or indirect carcinogenic effect by either encoding viral oncogenes which plays a role in the transformation of cells or causing persistent infection which results in chronic inflammation with significant tumorigenic effect. In most cases, virus do not directly initiate the Oncogenic process but plays a role in the acquisition of the risk factors that are linked with the development of cancer (Fig 1) over many years because the process of cancer development involves series of multiple steps consisting of process that are linked with several mutations due to genetic damage[11,16] . Initiating of cancer by these oncoviruses is either via 1. Immune evasion where the cyclic GMP-AMP synthase associated stimulator of interferon gene (STING) , the retinoic acid-inducible gene 1 (RIG-1), and Toll-like receptor (TLR) pathways which are associated with immune responses through sensing of DNA and RNA viruses are inhibited[16,17] ; 2. Abnormal regulation of cell cycle that target tumor protein 53 (p53) and retinoblastoma (pRB). These are tumour suppressor genes (TSG) are mostly found in tumour cases. These oncoviruses code for oncoproteins that have the ability of degrading or repressing p53 and pRB[17, 18]; and 3. Oncogenic viruses can also target series of host signalling pathways that controls cell growth and expansion; for e.g. mitogen-activated protein kinase (MAPK) pathway. In most cases, oncoviruses initially down regulates TSG or activate oncogenes that are associated with these pathways [17, 19].

In tumour development, various noncoding RNAs are found to be dysregulated, which in many cases are suspected to drive either tumourigenesis or metastasis. Close correlation have been reported between patient outcomes and levels of certain long noncoding RNAs in their tumours. Some noncoding RNAs make their way into the bloodstream thereby presenting unique opportunities for the diagnose of disease, making inform treatment decisions and also monitor patients' response to therapy with simple blood test. Individual RNAs or RNA profiles may therefore be useful as diagnostic tools to help determine the best treatment approach for the individual patients. In addition, it will be possible to use mRNAs and siRNAs as potential therapeutic targets. One promising area is the development of short pieces of synthetic RNA for cancer treatment and mRNA for development of cancer vaccine. These molecules, also known as antisense oligonucleotide, can be designed in the laboratory to bind specific RNAs inside cells and in so doing, activate or turn on RNAs. In some cases, antisense oligonucleotide can be used to inhibit the production of proteins that are essential for the growth of certain cancers. For e.g. BART-encoded miRNAs are abundantly expressed

in epithelial tumours; hence, these miRNA may serve as diagnostic and prognostic biomarkers for EBV-associated malignancies such as BL and NPC[84-86].

Approximately 15-20% of human cancer cases are associated with viruses [1-3] with the data strongly supporting the evidences that several viruses are carcinogenic[4]. The association of a virus to a specific cancer is approximately between 15% and 100%[18]. According to the International Agency for Research on Cancer (https://gco.iarc.fr/causes/infections/help), ten pathogens are associated with cancers: H*elicobacter pylori*,), *Opisthorchis viverrini, Clonorchis sinensis,* and *Schistosoma haematobium.* These are non-viral agents. The viral onco-agents are hepatitis B virus (HBV), hepatitis C virus (HCV), human Papillomavirus (HPV; types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are collectively referred to as high-risk types while 6, 11,42,43,44,53,54,61,72, and 81 are collectively referred to as low-risk types , Epstein–Barr virus (EBV), human herpesvirus type 8 (HHV-8; also known as Kaposi sarcoma-associated herpesvirus), human T-cell lymphotropic virus type 1 (HTLV-1). *Helicobacter pylori* cause most cancer followed by HPV[12]. Cancers for which there has been strong established evidence of association with these pathogens include human Papilloma virus (HPV), Kaposi's sarcoma herpes virus (KSHV), human T-lymphotropic virus 1 (HTLV-1); Epstein Barr virus (EBV), Hepatitis B virus (HBV); Hepatitis C virus (HCV); Merkel cell polyomavirus (MCPyV), and human cytomegalovirus (CMV) have all been associated with various form of cancers[4-7, 13]. However, HIV needs to be added as it has been associated with AIDS-defining and –non-defining cancers[83]. Cancers due to viruses are of public health concern, for e.g. with HPV, globally, 625,600 women and 69400 men are getting HPV-associated cancer each year[14]. Table 1 shows some viruses that possesses oncogenic capabilities.

The aim of this article is to provide a through and current review of the current state of knowledge of biomarkers in EBV-associated malignancies and highlight the importance of biomarkers in the diagnosis, prognosis, and therapy in EBV-associated cancers.

Table 1: Major viruses associated with cancers

Virus	Disease	References
EBV	**BL, NPC, HL ENKTL-NT, NKTL-NT, EBVaGC**	**12, 31**
HBV and HCV	**HCC**	**20,21**
HTLV-1	**ATL, cervical cancer, skin cancer in patients with EV Head and neck cancer**	**22**
HHV-8	**Kaposi's sarcoma, primary effusion lymphoma, Castle's disease**	**23**
HERV	**Germ line tumours, breast cancer, ovarian cancer, melanoma**	**24,25**
HPV	**Cervical cancer, skin cancer in patients with EV, head and neck cancer, other anogenital cancers**	**20,21**
HIV	**Hodgkin's lymphoma , skin-,lung-, anal-and kidney-cancers**	**83**

Abbreviations: EBV, Epstein-Barr virus; BL, Burkitt's lymphoma; NPC. Nasopharyngeal carcinoma; HL, Hodgkin lymphoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ATL; adult T-cell leukemia; HTLV-1, human T lymphocytes virus-1; EV, epidermpdysplasia verruciforms2; HHV-8, human herpesvirus-8; HERV, human endogenous retroviruses; HPV, human Papillomavirus; ENKTL-NT; extranodal NK/T-cell; ENKTL-NT, nasal type; EBVaGC, EBV-associated gastric carcinoma; HIV; human immunodeficiency virus

Clinical Biomarkers

Biomarkers (also referred to as biological markers) can be defined as "molecules that help to indicate the biological state, process, event or condition"[8]. The World Health Organization (WHO) defined biomarkers as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction [9]. In healthcare, biomarkers are scientific markers that indicate health disorder or other biological state of an individual. Furthermore, it can be an indicative of biological process, disease process or a response to therapeutic interventions. Biomarkers are being utilized in all facets of sciences such as chemistry, physics and biology. When narrowed to medicine, biomarkers can be referred to as specific protein level in blood which could be used to measure the presence, progression or severity of a disease and influence therapy. For the past decades, biomarkers have been sparingly used in medicine, agriculture,

etc. However, as a result of scientific advances, there has being increased utilization of biomarkers especially in healthcare. Biological markers can facilitate early detection of a disease stage and be targeted to improve diagnosis, prognosis, and better treatment of a particular disease. Recent advances in omics and high throughput has been an essential addition in the field of biomarkers research and development. There are two major types of biomarkers[10]: Biomarkers of exposure which are used for risk prediction and biomarkers of disease used for screening and diagnosing as well as monitoring disease progression. Therefore based on their characteristics, these major biomarkers can be grouped based on different features such their characteristics, genetic and molecular biology, and clinical application (Fig 1). An ideal biomarker should be reliable, safe and easy to measure, sensitive and specific, cost efficient for follow-up, are modifiable after treatment, and be important in diagnostic, prognostic and evaluating outcome and/ or stage of diseases. These features are important in utilizing biomarkers for diagnostic, prognostic and evaluation of therapeutic responses. Biomarkers are associated with some benefits and drawbacks which should be taking into consideration before their utilization in the clinical environment. Table 1 outlines some of these benefit and drawbacks.

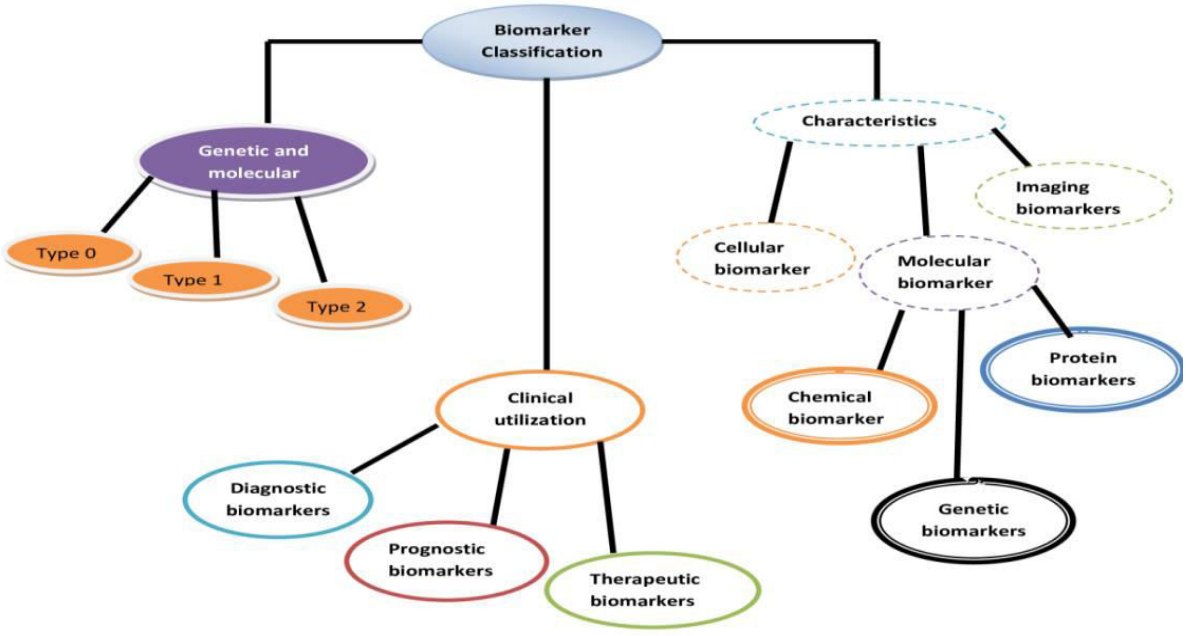

Fig 1: Graphical representation of classification of biomarker. The genetic and molecular biology is divided into 3 types: type 0 is the natural history of the biomarker, type 1 is therapeutic activity biomarker, and type 2 is surrogate biomarker which is regarded as the clinical outcome of disease and prediction for therapeutic response[76].

Table 1

Advantages and disadvantages of biomarkers

Advantages	Disadvantages
Measurement is precise	Timing important
Economical	Expensive
Less bias when compared to questionnaires	Sample needs to be stored for long
Rapid warning signal	Normal range difficult to establish
Reliable	Ethical responsibility needed
Validity can be established	Laboratory errors common
Disease mechanism can be studied	
Objective assessment	

The development of biomarkers consists of several steps that start with the discovery of the biomarkers in diseased and healthy individuals. As suggested by Bodaghi et al, biomarker development involves the following steps: pre-analytical and analytical validation, clinical validation, regulatory approval, and demonstration of clinical utilization. After the discovery of a novel biomarker, statistical tools such as multivariate statistical analyses, Principal Component Analysis (PCA), Random forests, Classification and Regression Tree, and Ranking PCA are used to assess and evaluate the biomarkers. Fig. 2 outlines the steps used in identification and optimization of a novel biomarker

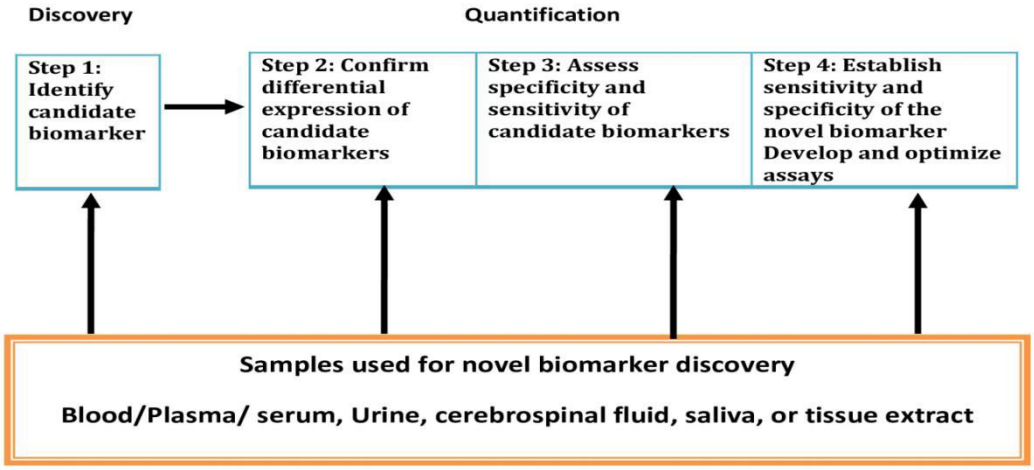

Fig 2: Steps in the development of biomarkers. Different bodily fluids such as whole blood, serum, plasma, and saliva/ tissue extract can be used.

Epstein - Barr virus (EBV)

EBV also referred as human herpes virus 4 (HHV4) is ubiquitous virus that belongs to the y-herpesviruses family. It consists of linear double stranded DNA of about 172 kilobases long. It was the first human oncovirues to be described[12]. It has been associated with several diseases including benign disease, oral disease, multiple sclerosis, oral hairy leukoplakia, various cancers such as haematological and epithelial cancers and EBV-related hemophagocytic lymphohistiocytosis[28, 31]. In 2020, approximately 378 000 new cancer cases were attributed to EBV with an estimated 209 000 EBV-associated cancer mortalities[29]. Primary infection is mostly through oral exposure during adolescent or childhood. Over the course of years, primary infection has been decreasing in the developed countries because of socioeconomic status such as higher income and education level is linked with the prevalence of lower age-specific antibodies[30, 31]. Transmission of EBV is through deep kissing, sexual intercourse, blood transfusion, allograft transplantation, and possibly close contact with carriers. The diagnosis of EBV is by heterophile antibody tests and/ or EBV-specific antibody tests. Although the public burden of EBV is high, we still do not have an effective licence vaccine or an approved antiviral agent for it; although several nucleosides have been reported to have some activities against EBV. However no clinical studies have proven its benefit. With interest in immunotherapy being generated in oncology, immunotherapy approach may be ideal interventions for EBV-associated cancer.

EBV is associated with several cancers (Table 1) and risk factor for EBV-associated cancer is attributed to geography-specific distribution with some of the distribution linked with phylogeographic distributions between the different strains of EBV[29, 31]. EBV is classified into EBV type 1 and EBV type 2 based on the genetic sequences of the virus. Globally, type 1 is more common while type 2 is linked with smaller burden of global infections. However, type 2 is frequently found in Africa, Alaska, and Papua Guinea in comparison to America, Asia, and Europe [29]. Co-infection and immunocompromised status are also risk factor for acquiring EBV. A study found that EBV type 1 was significantly associated with infectious mononucleosis[32, 33]. Type 1 list common cancers associated with EBV[29, 34]. However, a study found significant association between EBV type 2 and risk of NPC although EBV type 1 was prevalent in the region. Also others linked EBV to HL and EBVaGC[29]. Ethnicity and country of birth are risk factors for developing EBV-associated NPC. This association was linked to

lifestyle factors such as dietary and genetic factors such as single nucleotide polymorphisms in human leucocytes antigens[35]. Risk factor for EBV-associated cancers is multifactorial which are dependent on environment and genetic factors. More research is needed to elucidate the pathomechanisms of EBV-associated cancers.

The hallmark of cancer (Fig 1) is uncontrolled proliferation of cell in which abnormal genes present in cancer cells directly play a role in regulating cell cycle. The primary duty of cell cycle is to promote DNA replication one time during S phase and then similar chromosome copies are then dispersed equally in two daughter cells in M phase. Cell mitosis then takes place as the cell transverse through a restriction point (R) later in G1 after which they enter S phase. Defectiveness in cell cycle leads the cell to grow without control which is characterized by primary hyperpropagation and apoptosis which subsequently leads to carcinogenesis[36].

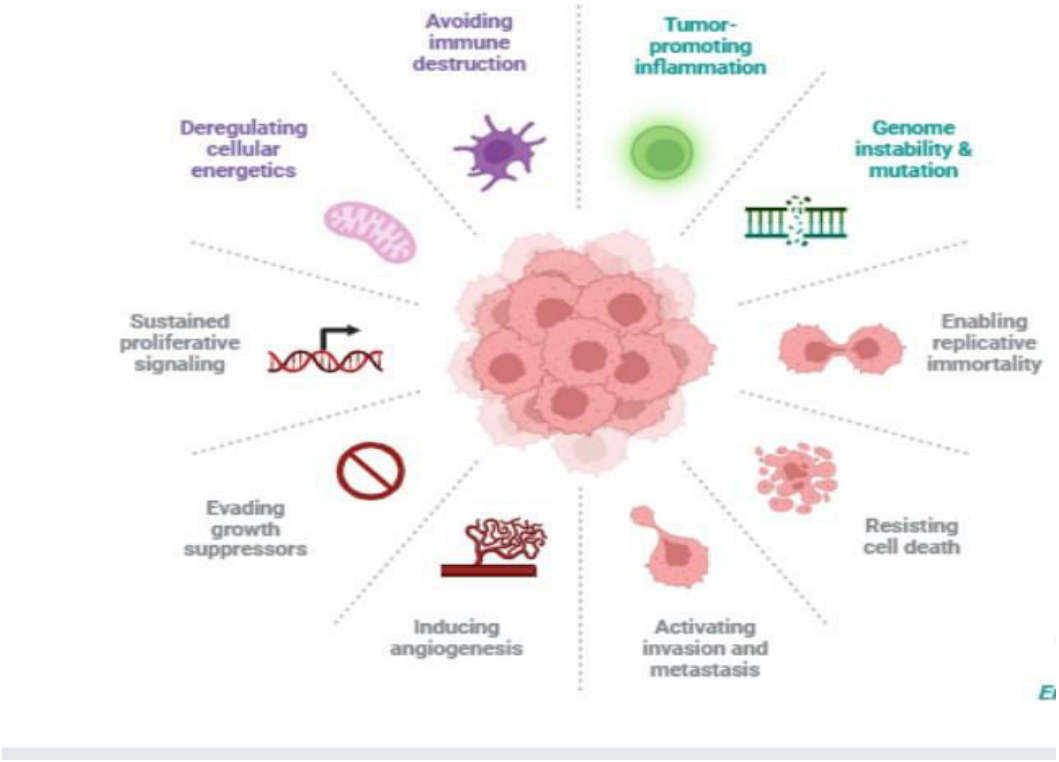

Figure 2: Hallmark of cancer (Source: Biorender, 2023)

After primary infection, EBV establishes two distinct life cycles (Fig 2) lytic replication and latent infection. During lytic replication which takes place in the oropharyngeal epithelial, there is viral replication which results in the production of multiple viral particles into the lymphoid tissue resulting infection of naïve B-cells[37]. EBV infects the B-lymphocytes resulting in long-term persistence of the virus. The memory B-cells are colonized by the virus resulting in latent infection which is characterized by the expression of latent genes. During latency, the viral genome is preserved as nuclear episomes in which only few EBV genes are produced[38-40].

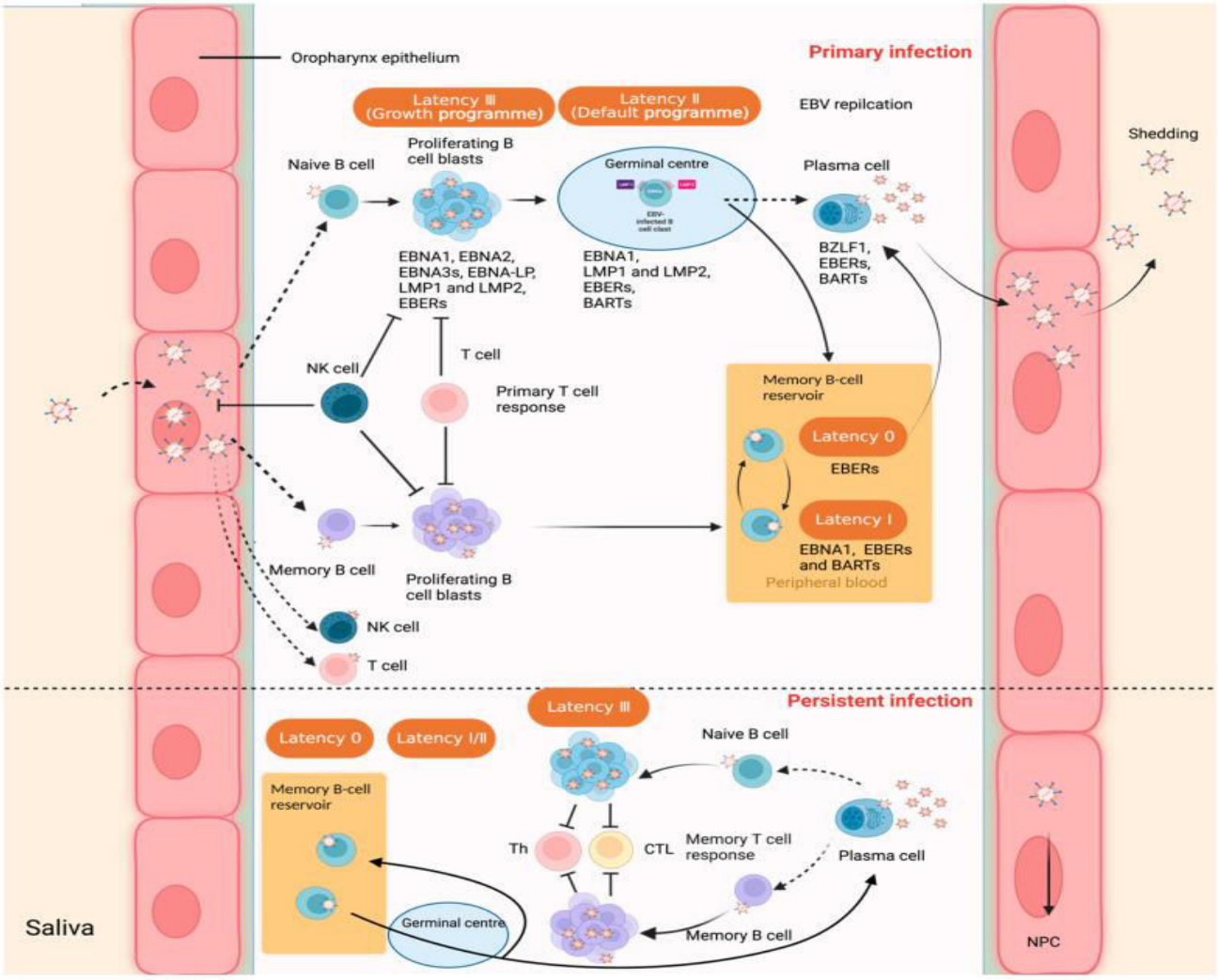

Fig 3: Molecular interactions involved in EBV life cycle in B lymphocytes (Source: 28)

Table 2

Latent genes associated with EBV-associated malignancies

EBV latent type	Genes	Disease
I	**EBNA-1, EBER**	**BL**
II	**EBNA-1, LMP-1, LMP-2A and 2B, EBERs, BARTs**	**NPC, HL, GC, NK/T cell lymphomas**
III	**EBNA-1, LMP-1, LMP-2A and 2B, EBNA-2, EBNAA, B,C and LP, EBERs, EBV-miR-BHRF1/BARTs**	**AIDS-related NHL, partial BL, PTLD, DLBCL**

Biomarkers of EBV-associated Malignancies

Biomarkers associated with malignancies can be found in whole blood, plasma, serum, urine, stools and other discharges such as cervical or nipple discharge or other human biological fluids. They can therefore be assessed without invasive technique or can be tissue-derived which require either biopsy or surgical resection. An ideal biomarker for cancer should possess the following features: 1. Produced by the tumour; 2. Should be linked with the burden of the tumour; 3. Be detected in preclinical or early phase in human biological fluids such as whole blood; 4. Not be detected in healthy individuals; 5. Be easily measured; and 6.Have high analytical specificity and sensitivity so that it can be used to differentiate those with the tumour from those who do not have it. Below are some predictive biomarkers of EBV-associated malignancies.

Viral load as biomarkers of EBV-associated malignancies (EAM)

Viral load is a very important marker that can influence the development and progression of disease[41]. In severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) viral load was associated with increased severity and deaths in patients affected with the virus[42]. In the study it was suggested that SARS-CoV-2 load was predictive of SARS-CoV-2 associated mortality. Because viral DNA is present in all stages of EBV-associated malignancies, this can be used as biomarkers for diagnosis and prognosis. In nasopharyngeal cancer (NPC) associated with EBV, a study suggested that in patients who are positive for EBV-DNA, treatment with intent to cure having EBV-DNA load of >70 copies/cell is linked with better outcome based on seven years disease-free survival[43]. Previous studies used EBV-DNA load to screen for NPC and as diagnosis tool for early cancer stages as well as evaluating

treatments effects[44, 45]. Furthermore, EBV-encoded small RNAs are linked with better outcome when compared with negative cases[46]. Another study suggested that EBV-DNA load can be used as a diagnostic tool in patients with EBV-associated NPC who have advanced T tumours especially those with T1-T2 tumours[48]. However, a study didn't identify any correlation between viral status and tumour stage[47]. EBV-DNA load can also be used in evaluating therapeutic interventions. A single-arm multicenter phase 2 study (termed POLARIS-02 study) evaluated the antitumor activity, safety, and biomarkers of toripalimab, a programmed death-1 (PD-1) inhibitor against NPC. They found that decrease in EBV-DNA load in plasma was associated with favourable response[51]. Quantification of EBV-DNA load can be used as important tool for diagnosis, prognosis, and evaluating the effects of therapeutic interventions. However, large cohort studies are needed to validate this suggestion.

The specimen to be used for measuring viral load is important. Blood compartments such as unfractionated whole blood, serum, plasma, and isolated peripheral blood leukocytes and mononuclear cells have been used. Unfractionated whole blood should be the ideal specimen for such measurement. Stevens et al suggested that whole blood specimen should be used instead of serum for EBV-DNA load measurement as using serum leaves out circulating cell-associated virus and may lead to an overestimation of the load in the specimen[49]. This finding was consistent with earlier study that measured EBV-DNA load using whole blood in lung transplant recipients is a predictive and diagnostic tool for post-transplant lymphoproliferative disorder (PTLD) [50]. This means measuring EBV-DNA load can be used as marker of latently infected B-cell. However, others have suggested using plasma as a tool for measuring EBV-DNA load in early stage of NPC[44]. Molecular techniques such as real-time polymerase chain reaction (PCR) can be used as a tool for such measurement. PCR has been used globally for diagnosis and management of diseases. This can be utilized in EBV-associated cancers. However, due to variation in the clinical specimen used around the globe, standardization of measuring EBV-DNA load using blood compartment is required for universal comparisons.

Immunological Profile as Biomarker of EBV-associated Malignancies

Immune profile can be used in predicting outcome of EBV-associated cancers and other cancers. The immune system can serve as marker of an individual's response to the presence of cancer cells such as EBV-associated malignancies. Chronic inflammation is a factor in the

development of cancer. Chronic inflammation promotes the process of malignancy development via the induction of growth factors, inhibition of growth resistance and apoptosis then initiates malignancy development through angiogenesis and metastasis[54]. Cytokines are used to study inflammation; therefore cytokines can be predictive biomarkers of inflammation in EBV-associated chronic infection which might lead to the development of EBV-associated malignancies. Profiling cytokines is a good technique for measuring the process of inflammation[52]. Cytokines can be used to evaluate the nature of assault, injury, infection, and utilized in elucidating the stages of disease process[53]. One key factor that regulates chronic inflammation is inflammasomes which are intracellular multi-proteins essential for initiating immune response through activation of inflammatory caspase, especially caspase-1. It then elicits the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 as well as tumor necrosis factor-α (TNF-α) as signatures in tumour microenvironment (TME) leading to induction of pro-inflammatory response[55]. IL-1β and IL-18 can therefore be used as biomarkers of chronic inflammation in EBV-associated infection which leads to carcinogenesis. In NPC, activation of nuclear factor kappa B (NF-Kβ) signalling is associated to better survival and tumour development via up regulating the expression of oncogenes and anti-apoptotic genes[55]. This suggests that NF-Kβ can be used as marker of survival and outcome of carcinogenesis in EBV-associated tumours. Other cytokines such as TNF-α, IFN-y IFN O y-10, TGF-β, RANTES, TARC, TNF-α and CCL28 can be used as markers of prognosis, diagnosis and therapeutic effect in EBV-associated malignancies[56, 57]. Immunotherapy intervention such as immune check inhibitors (ICIs) has been suggested for cancers; such as EBV-associated gastric cancer. To evaluate the efficacy of such intervention, programmed cell death ligand 1 (PD-L1), microsatellite instability-high (MSI-high), DNA mismatch-repair deficiency (dMMR), and tumour mutation burden (TMB) can be used as markers to evaluate therapy[58, 59]. However, care must be taken as these treatment-associated biomarkers may have certain limitation; for e.g. patients lacking these markers may lose any therapeutic benefits. More studies are needed to develop universal guidelines for using these cytokines in viral-associated cancers. In addition, researches are needed to develop predictive biomarkers for ICIs resistance in all virus-associated cancers. Cytokines can be profiled with serological assay such as enzyme-linked immunosorbent assay (ELISA) while the efficacy and predicting ICIs and other immunotherapy can be evaluated with next generation sequence (NGS) and immunohistochemistry. The role of multiomics as means of identifying biomarkers will be discussed later. In general, the specificity and sensitive of each technique should be validated before use.

EBV oncoproteins as biomarkers of malignancies

EBV-associated oncoproteins are the drivers of tumour development in EBV-associated cancers. Because of their importance in EBV-associated cancers, these oncoproteins can be used as markers for diagnosis, prognosis, and therapeutic targets. Oncoproteins have been described in EBV-associated - BL, -NPC, and -gastric cancer, EBV nuclear antigen 1(EBNA1), small non-coding RNAs (BAMHI A rightward transcripts (BART), and EBV-encoded small RNAs (EBERS) while in HL, NPC, T/NK lymphomas, EBER, BART, EBNA1, latency membrane protein 1 (LAMP1) and LMP2 genes have been also been reported[60] . These proteins have effects on cellular pathways and growth. For e.g. LMP1 is one of the LMP oncogenes needed for EBV-elicited B-cell immortalization *in vitro* and most importantly possess oncogenic ability in non-lymphoid cells, facilitates the induction of growth transformation in some immortalized fibroblasts cells from rodents[63]. LMP1 and LMP2 cooperate to promote the development of carcinoma in mouse carcinogenesis models through activating multiple signalling pathways[64]. Although it has been suggested that LMP2 has a role in malignancy, the data is not conclusive. However, LMP2-specific antibodies have been consistently found in NPC patients and in HL as well as NPC tissues[65, 66]. This suggests the role of LMP2 in development of malignancies. These oncogenes are potential markers of EBV-associated cancers. In EBV-associated NPC, Wang et al reported that expression of LMP1 and BARF1 in tissue samples were associated with tumour-node-metastasis stage and gastric cancer[61]. MiR-21-5p is one of the most described microRNAs associated with oncogenesis in EBV infection. Kolesnik et al found significant high level of miR-21-5p among patients with EBV-associated oropharyngeal cancer when compared with EBV-negative individuals[62]. MiR-21-5p can therefore be used as biomarker of diagnosis and therapeutic intervention for EBV-associated oropharygneal cancer and other cancers. EBNA1 is the only oncoprotein that has been detected in almost every EBV-associated malignancy. EBNA1 is associated with survival in BL. It can therefore be a predictive biomarker in all EBV-associated malignancy. EBNA-3A, 3B, and 3C are proteins that regulates viral and host transcription. EBNA-3B and -3C have been found in B-cell transformed cells. With EBNA-3A, their function in cellular transformation has not been clearly elucidated because when viruses lacking EBNA-2C were used to infect B-cell, there was inhibition of growth and initiation of apoptosis[63]. TSG can also be potential biomarkers of malignancies with hypermetylation of TSG linked with carcinogenesis. In NPC, it has been shown that such

hypermethylation resulted in the loss of heterozygosity (LOH) on 3p, 9p, 11q and 13q chromosomes which was associated with early activities of carcinogenesis[71]. In addition, LOH on 3p chromosomes was found in approximately 100% of NPC cases and 75% premalignant lesions. It was therefore suggested that chromosomal changes is associated with development and progression of NPC and other cancers. Therefore TSG markers such as p16, p15, Ras Association Domain Family 1A (RASSF1A), Death-associated Protein Kinase (DAP-Kinase), CDH1, E-cadherin, HIN-1, MGMT, MLH, WIF1, etc can be used as biomarkers for various purposes. For e.g. p16 is one of the most recognised inactivated tumour suppressor genes in human cancer which is an inhibitor of cyclin-dependent kinases with the ability of reducing cell cycle through rendering progression from G1 phase to S phase[73] . Therefore p16 promoter hypermethylation can serve as ideal biomarker of diagnostic that can differentiate malignant malignancies from non-malignant tumours. WIF1 is a protein that binds lipids to Wnt proteins thereby preventing them from triggering the signal pathway. This protein can be used for both diagnosis and prognostic purposes in cancer especially in EBV-associated malignancies. RASSF1A is a novel biomarker for early diagnosis of cancers such as NPC; with several studies reporting of presence of RASSF1A promoter hypermethylation in many primary cancers including NPC[71]. However, it must be noted that single epigenetic alterations cannot be used as basis for diagnostic and prognostic purposes because such changes are not sensitive enough to serve these purposes. This means focusing on single epigenetic changes can result in false-negative results. Utilizing multi-purpose assay can address this limitation. In a study, Nawaz et al used multiplex methylation-specific PCR biomarker assay for early detection and follow-up of EBV-associated NPC[74]. In their study, the assay included three TSGs: ITGA9. RASSF1A and p16 which was incorporated with the following oncoproteins: EBNA1 and LMP1. Multiplex utilization of TSGs combined with oncoproteins would be an ideal approach for both diagnostic and prognostic purposes in cancer cases as reported by Jiang et al who showed that multiplex assay consisting of the following hypermethylated TSGs: WIF1, UCHL1, RASSF1A, CCNA1, TP73, and SFRP1 in NPC was associated with poor disease-free survival[75] . While these oncoproteins and TSGs can be used as potential biomarkers in EBV-associated malignancies, more studies are needed to validate them. Also a global consensus is needed on the sensitivity and specificity of using them as biomarkers of diagnosis, prognosis and evaluation of treatments.

Circulating DNA (ctDNA) as biomarker of EBV-associated malignancies

Circulating DNA (ctDNA) is cell-free DNA which resulted from the lysis of cancer cells or active secretion. It is due to genomic changes similar to those observed in tumour DNA. In viral associated malignancies, ctDNA is due to apoptosis, necrosis, or generated as a result of viral replication. When compared with healthy and cancer patients, there are difference in ctDNA profile making ctDNA as a good biomarker of cancer development and progression[67]. ctDNA can be identified in body fluids such as saliva, urine, and blood. ctDNA was used in evaluating therapeutic response among patients with metastatic gastric cancer and EBV-positive tumours. Significant response was observed when patients were given pembrolizumab with changes in ctDNA associated with improved positive outcome[68]. EBV-associated DNA has been shown to be of clinical importance for prognosis, surveillance, and screening for NPC[69]. ctDNA was observed in EBV-associated NPC. Lam et al reported of differential characteristics of plasma EBV DNA between NPC and non-NPC patients with higher concentration and longer fragments size of plasma EBV DNA[70].

Circulating ctDNA profiling would be useful blood-based biomarkers for EBV-associated malignancies and it has one advantage: it is non-invasive meaning it can be widely used. Within the clinical setting, ctDNA profiling can be used for evaluating the prognosis of cancers

However its usefulness can be limited by very low concentration of ctDNA in the bodily fluids which means very sensitive tools are required for their detection. In addition, protocols are required that promote single time blood sampling instead two time screening. This is because two times screening is associated with anxiety as patients wait for follow-up tests. There would be logistic challenges such as cost of recalling patients for second tests while compliance maybe become another issue in clinical settings. Since ctDNA profiling can become important biomarkers for virus-associated malignancies, it will be essential if the fragment patterns of plasma circulating viral DNA are elucidated in different EBV- and viral-associated malignancies. It is therefore important that short plasma DNA molecules are preserved in the lab during procedure during sample preparation for ctDNA evaluation as this would improve diagnostic performance. Also, validation and clinical trials should be undertaken to evaluate this blood-based biomarkers for clinical use. Finally, ctDNA blood-based sample tools would be useful for diagnosis, prognosis, and therapeutic responses in viral-associated malignancies.

Multiomics in EBV-associated cancer biomarkers discovery

As a result of technological advancement, multiple biological layers can be studied at the same time, ranging from genome, transcriptome, proteome and metabolome profiling. These integrative evaluation leads to data that gives an insight into the biological system been evaluated. Multiomics approach is been used across many spectrum of biological settings including cancer biology, host-pathogen biology, microbiology, and regulatory genomics[77]. Multiomics is the combination of different "omics" that consist of genomics, proteomics, transcriptomics, and metabolomics. Table 3 outlines some characteristics of the available omics

Table 3

Characteristics of the available omics

Types	Aim	Techniques use
Genomics & epigenomics	**Characterize and quantify genes**	**GWAS, WES, NGS, SRM**
Transcriptomics	**Understanding the molecular changes induced by environmental factors or pathogenic agents**	**RNA-Seq** **Probe-based arrays** **NGS**
Proteomics	**Maximum identification and quantification of all proteins in cells or tissues**	**2D- DIGE. LC-MS, MALDI-TOF, TMT, SRM** **MS-based assays** **High throughput assay** **Phage display assay** **Yeast tow-hybrid assay** **Affinity purification assa**
Metabolomics	**Studying set of small molecules metabolites derived from cellular and biological processes**	**Mass spectrometry. NMR spectroscopy**
Microbolomics	**To study the ecological community of microorganisms that lives on plants or animals**	**16S rRNA gene sequencing** **NGS assay** **Metagenomics (e.g. QIIME)**
Radiomics	**Using high-throughput imaging to aid in the diagnosis, classification or grading of diseases**	**PET, MIR, CT**

Abbreviation: GWAS, genome-wide association studies; WES, whole-exome sequencing; NGS, next generation sequencing; SRM; selected reaction monitoring; 2D-DIGE, 2D differential gel electrophoresis; LC-MS, liquid- mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-F; TMT, tandem mass tag; NMR, magnetic resonance imaging; PET, positron emission tomography; MIR, magnetic resonance imaging; CT, computed tomography; QIIME, quantitative insights into microbial ecology

Multiomics techniques have been used in different facet of human diseases. This has led to development of data on genome, proteome, transcriptome, and metabolome that aids in choosing markers and targets for in human diseases. Single omic techniques aids in providing useful knowledge however multiomics approach is more promising. A review by Boron et al found that in endometrial cancer (EC), the following biomarkers were identified: tomoregulin-2 which was target for early diagnosis and treatment of EC; ring finger protein 183 which could serve as prognostic and early diagnostic markers for EC; zinc finger protein 558 as prognostic biomarker, LGR and PTGDS as biomarker and therapeutic target[77]. Genome, transcriptome, proteome and epigenome techniques were utilized in most of the studies included in their review.

Bao et al used multiomics analysis to identify $S1009^{+}$ as potential immune therapeutic target for colorectal cancer (CRC) [78]. S10049 is a damage-associated molecular patterns receptor that has been shown to be important in regulating the function of macrophage. Their study was based on integrated transcriptomic, proteomic, and metabolomic analysis. Tumour tissues and plasma samples; tissue samples used for transcriptomics and proteomics while plasma samples were used for metabolomics. In CRC, three types of immunometabolism subtypes have been described as C1, C2, and C3 with C3 showing worse prognosis in CRC. The expression of S10049 can dedifferentiate C3 from non-C3 subtype macrophages which suggest that s10049+ is an important immunotherapeutic target for C3 patients because these patients showed poor outcome and did not respond properly to immunotherapy.

Saha et al by using multiomics suggested that the kidney-type glutaminase (GLS) and liver-type glutaminase (GLS2) could be used as prognostic markers in clinical outcomes of cancers as well as therapeutic targets for cancer. GLS and GLS2 are dysregulated in many cancers. GLS was associated with poor outcome in breast, head and neck, leukemia, and oesophagus tumours while GLS2 was associated with poor outcome in colon, blood, ovarian, and thymoma tumours[79]. Since GLS and GLS2 are associated with different cancers, they can also be evaluated as potential prognostic biomarker for EBV-associated malignancies.

Xiao et al also used integrative multiomics technique to evaluate the role of metastasis-related gene (MRG) as a prognostic marker for breast cancer (BC) [80]. They reported that EZR was found to be significantly increased in BC since it was expressed in both BC cells and BC tissues. When EZR was knockdown, it led to inhibition of cell proliferation, invasion and chemoresistance in BC. EZR is an oncogenic gene which is known to be involved in cell

adhesion and migration in cancers. EZR can therefore be used a novel prognostic biomarker for BC associated with EBV. It should also be evaluated in other types of cancers that are associated with EBV. Other prognostic markers that have been reported in BC include matrix metalloproteinase 2, CD44, and MDM2. Multiomics can also be used in identifying biomarkers of adverse event during therapeutic interventions for cancers associated with EBV. Jing et al used multiomics to predict immune-related adverse events during checkpoint immunotherapy[81]. They found that LCP1 and ADPGK were predicted immune-related adverse events when patients were given anti-PD-1 or anti-PD-L1 immunotherapy. Finally, Zhao et al using multiomics identified survival-related genes which were associated with prognosis in many cancers. These include SLK, AP15, BTBD2, PTAR1, VPS37A, EIF2B1, and ZRANB1.

Taking these data into consideration, it can be suggested that malignancies-associated genes (MAGs) in EBV-associated tumours can be evaluated as novel biomarkers for diagnostic, prognostic, and therapeutic responses. Table 1 presents some multiomics software used for integrative analysis of data.

Table 4

Some resources for multiomics data integration

Method	Uses	Tool Link
3Omics	Analysis, integration and visualization of human transcriptomic, proteomic, and metabolomic	https://3omics.cmdm.tw
trackViewer	Interactive and integrative visualization of multiomics data	https://bioconducter.org/packages/release/bioc/html/trackViewer.html
VANTED	Integrative	https://www.cls.uni-konstanz.de/software/vanted

	visualization and analysis of omics data	
ivTerm	Interactive visualization of functional analysis of multiomics data	https://github.com/SJTU-CGM/ivTerm
motifStack	Analysis of transcription factor binding site evolution	https://bioconducter.org/packages/release/bioc/html/motifStack.html
PaintOmics	Integrative analysis of multiomics data	https://www.paintomcs.org
ChIPpeakAnno	For annotating ChIP-seq and ChIP-chip data	https://bioconducter.org/packages/release/bioc/html/ChiPpeakAnno.html
LinkedOmics	Analyzing multiomics data in oncology	https://www.linkedomics.org

Conclusion

Cancer is a complex disease which involves mutation in the genome and oncoviruses have been found to be associated with genomic mutation, although they are not directly involved in the carcinogenesis process. Just like other cancers, EBV-associated malignancies are of global importance with glaring knowledge gap. One of the important knowledge that would help in proper management of EBV-associated malignancies is developing biomarkers of diagnosis, prognosis and therapeutic responses. In addition, these novel biomarkers can be

evaluated for therapeutic targets. However, this biomarkers must to be validated and standardized before adopted for international utilization. Future research should target at discovering biomarker of immunotherapy response in EBV-associated malignancies. However, such studies would be limited by sample size, which may need many years of multiple centre collaboration. However, robust and highly efficient techniques would be required for the development of EBV- and viral-associated malignancies biomarkers. In order to validate these biomarkers of adverse events, large sample sizes are essential requirements for identification of immune associated adverse events for identification of biomarkers of therapeutic interventions. Several techniques are being utilized for discovering of biomarkers.

Multiomics approach is a novel approach that can be used for identification of biomarkers in EBV-associated malignancies. Multiomics has been used in endometrial cancers to select series of biomarkers and therapeutic targets[77]. This approach can therefore be used in identifying therapeutic targets in EBV- and other viral-associated cancers in addition to its predictive roles in diagnosis, prognosis, and evaluation therapeutic responses.

Search Strategy and Selection Criteria

To identify articles on biomarkers of oncoviruses, we searched PubMed and Scopus using the search terms. Only articles in English were considered. The search ran between 05/10/2023 and 30/11/2023. We supplemented these searches by reviewing citations in identified manuscripts, recent conference abstracts, and our understanding of the subject. Because this is a narrative review, we didn't undertake meta-analysis as we deemed it not necessary.

Conflict of interest

The authors do not have any conflict of interest to declare

Disclosure

The authors have no disclosure to declare

Use of Artificial Intelligence

The authors confirm that no aspect of artificial intelligence was used while writing this article

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