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Chapter 3

Breast Cancer as an Epstein-Barr Virus (EBV)-Associated Malignancy

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Abstract

The Epstein Barr Virus is among the very first oncogenic viruses to be identified as culprits of human malignancies. Its role as an etiologic agent of breast cancer however remains debated despite mounting molecular evidence. In this chapter we address the challenge of multiple molecular etiologies of breast cancer (BC) with emphasis on the Epstein Barr Virus (EBV) as a potential causative agent within a frame work of gene/environment interaction. We also hope to contribute to a critique of the a concept of universal single agent or gene in cancer etiology. In addition to reviewing further reasons of why EBV should be considered a tumor virus, coupling molecular targets at the initiation stage, we examine evidence for the culpability of EBV as oncogenic virus in relation to the genetic and epigenetic events that leads to carcinogenesis of cancer; and the subsequent downstream interaction including genetic and epigenetic modifiers of signaling and molecular function underlying the cancerous phenotype. The TNF family is taken as an example of how the epigenetic reprogramming process, impacts molecular targets and how these combined interplay of molecular events impinges on pathogenesis and malignancy of breast cancer in humans.

Keywords: Epstein-Barr virus, breast cancer, genetics, epigenetics, microRNA, tumor necrosis factor

1. Introduction

1.1. Breast cancer etiology

Although the prevalence of breast cancer (BC) is relatively lower in sub-Saharan Africa compared to that of the "western" countries, it is characterized by aggressive nature and target

more women at a younger age [1]. BC etiology is not yet entirely understood, but its incidence is thought to be partially explained by environmental factors including viruses such as EBV [2]. Recently, a growing pile of evidence has accumulated with regard to the association of cancers and viruses. Viruses are believed to cause from 15 to 25% of all malignancies and this percentage will increase by more than 50% in 2030 in developing countries [3, 4]. As transforming agents, viruses, seem ideal culprit in causing cell transformation. More recently, the virus was reported as a main culprit of breast cancer in Sudan [5]. A putative role for viruses was speculated based on the limited contribution of mutational events within tumor suppressors such as BRCA1, BRCA2, and p53 to breast cancer etiology [6]. Epigenetic silencing was also envisaged as an obvious candidate to entertain. The fact that methylation lies prominently at the interface of genes and the environment, and the known link between selfish DNA (viruses) and methylation makes it particularly important in understanding both short- and long-term evolutionary effects in oncology. Interestingly, in the same subset of BC tissues where fragments of the virus DNA were detected by *in situ* hybridization in nearly all samples, significant epigenetic silencing of tumor suppressers was observed in a limited but key set of genes such as BRCA1, BRCA2, and p14 [5].

1.2. Genetic and epigenetic modifiers in breast cancer

A transcriptome study for virus-host interaction identified few of the main partners of EBV in the host cell [7] as of oncogenic potential. This is essential for a framework we are proposing in the current chapter. The framework (**Figure 1**) suggested by us and other authors [8, 9] entails the involvement of both genetic and epigenetic modifiers to converge on a cancer phenotype. However, we propose, in addition, an earlier role for the EBV virus in initiating that sequel of events through interaction of viral proteins and nucleic acid with key cellular components in the target cell (stem cell). Prominent among these cellular partners are RNA-binding proteins like ELAVL1/HuR, and editing genes like APOBEC/AID in the genomic side and DNMT, TET, and HDAC in the epigenetic side. One significant feature especially in the RNA-binding proteins is the plethora of potential targets and partners which could partly explain the wide spectrum of biological mechanisms involved and targeted by these events. Moreover, the virus molecular interaction could provide a plausible explanation to the features of organization described in previous publications [10] and increasingly ascribed to DNA/RNA editing and RNA-binding proteins like ELAVL1/HuR in addition to miRNA regulation. ELAVL1 has been reported to show marked centrality in a colorectal cancer family in which EBV infection is speculated to have a role [10]. The protein turned to display similar centrality among differentially methylated genes in breast cancer cases that had strong EBV positivity by *in situ* hybridization (**Figure 2**). This does not preclude a role for individual proteins like C-Fos, an established EBV partner [7], which has also been identified as independent predictor of decreased survival in breast cancer.

We simply try to differentiate between major upstream effecter molecules and downstream by-products of interaction like c-Fos and several other molecules; and cancer as a system and polygenic complex phenomenon, versus the rarity of a cancers of Mendelian-like monogenic inheritance where one or few molecules are key in determining a tumor phenotype.

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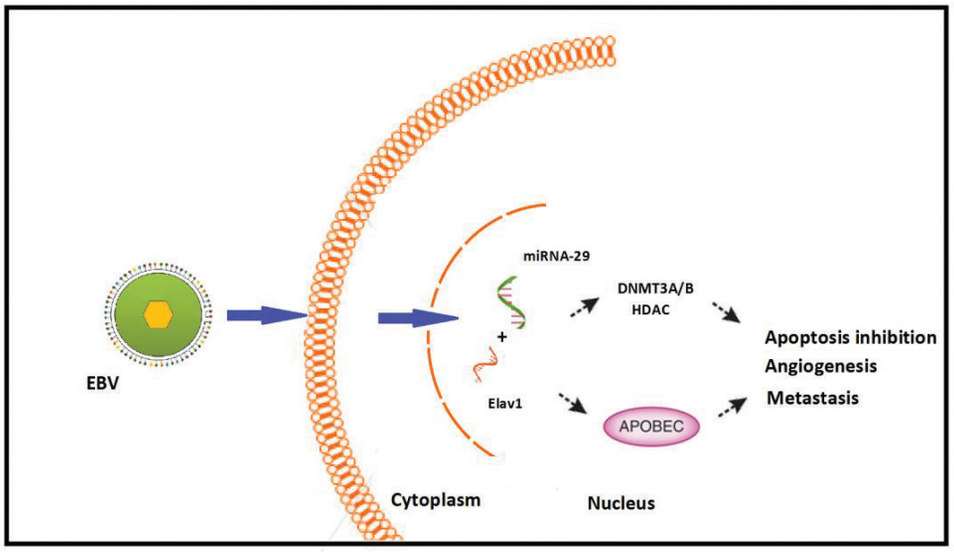

Figure 1. The oncogenic potential of EBV is outlined in the figure, where the interaction of viral proteins and nucleic acid (LNP, LNP2, BZLF, etc.) with key cellular components (ELAVL1/Hur, miRNA29) in the target cell (stem cell) dictates the consequent pathogenesis and carcinogenesis processes impinged by downstream molecules (e.g., APOBEC3) and involving both genetic and epigenetic modifiers.

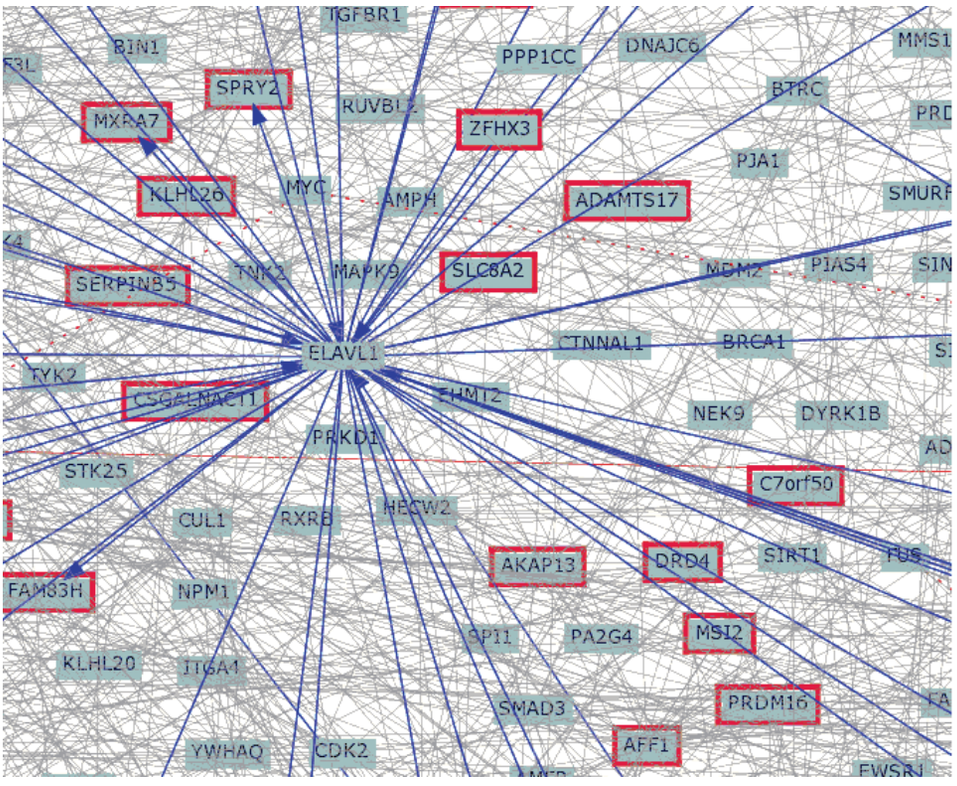

Figure 2. The centrality of ELAVL1/Hur is demonstrated through an interaction network of differentially methylated genes in breast cancer cases with strong EBV positivity using *in situ* hybridization from Sudan, using the program Cognosante.

2. Breast cancer and EBV

2.1. EBV infection molecular interaction and latency

EBV has been used routinely in laboratories to create cell lines for decades [11]. Furthermore, it has been found in breast tissue and is frequently found in breast secretions including breast milk [12]. EBV can infect mammary epithelial cells and its DNA fragment (p31) is capable of inducing immortalization in these cells [13]. This cosmopolitan γ-herpes virus infects usually at younger age. Its main target are B lymphocytes but it has a potential to infect epithelial cells as well and thus is associated with various lymphoid and epithelial malignancies and is incriminated as a carcinogenic agent by the World Health Organization [14].

EBV is closely associated with endemic Burkitt's lymphoma in sub-Saharan Africa [15] which earned the area the lymphoma belt due to such high frequency among children. The virus is associated with a horde of other malignancies in the tropics, such as nasopharyngeal carcinoma (NPC), gastric cancer, and breast cancer, although most studies regarding the controversial role of the virus as a cofactor in BC were done in countries outside of Africa. The variable prevalence of EBV in different regions is an indicator of the importance of the environmental and geographic cofactors in the development of such association and the diseases [16].

One key question to be entertained is why some oncogenic viruses like human papillomavirus (HPV) and EBV although common infections tend to develop cancer in some individuals whereas others remain asymptomatic? Should we speculate population-specific susceptibility factors that predispose to cancer in the human genome? Or whether some viral strains have more oncogenic potential as the case of HPV16, and 18 and EBV Type I, II and Type III? are there specific role and molecular basis of epigenetic silencing in inactivation of tumor suppressors, both of which environmental geographical cofactors play an important role in determining the strength of the association of malignancy with EBV [17] and hence variation in susceptibility may be influenced by factors such as geographical and immunological differences and ethnicity [18, 19].

The natural host of the virus is B-lymphocyte to which the virus gains entry through a type two complement receptor (CR2/CD21) [20]. Although breast cancer cells normally do not express the receptor CD21 [21], the range of viral tropism could be widened through the targeting of stem cells which are capable of expressing a wider range of receptor repertoires. EBV can infect primary mammary epithelial cells (MECs) that express CD21 and EBV infection leads to the expansion of early MEC progenitor cells with a stem cell phenotype, activates mesenchymal epithelial transition (MET) signaling and enforces a differentiation block. Hu et al. report that EBV can infect primary human mammary epithelial cells (MECs) but not tumor cells leading to phenotypic changes consistent with transformation [22]. Latent membrane protein-2A (LMP2A) may induce a stem cell state, evidenced by an enhanced self-renewal and transformational capacity, and also increases the number of tumor initiating cells *in vivo*, thus potentially rendering a B-lymphocyte into a cancer stem cell. This viral protein plays a key role not only in EBV latency and persistence but also in the progression of EBV-associated cancers such as NPC in which it was expressed in about half of the samples [23, 24]. It affects hedgehog signaling and induces stem cell behavior in epithelial cells [25].

When MECs were implanted as xenografts, EBV infection cooperated with activated Ras and accelerated the formation of breast cancer [22]. A human gene expression signature for MECs infected with EBV, termed EBVness, was associated with high grade, estrogen-receptor-negative status, p53 mutation, and poor survival. In 11/33 EBVness-positive tumors, EBV-DNA was detected by fluorescent *in situ* hybridization for the viral LMP1 and BXLF2 genes [22]. The observations that CD21 was absent on all of the tumor cell lines, none of which became infected, and that analysis of the TCGA breast cancer RNAseq data revealed no active transcription of EBV [26, 27] suggest that the EBV DNA detected in a subset of human breast cancers, is an inactive remnant of a previously active EBV infection that might have occurred in mammary epithelial cells years or even decades prior to cancer formation and which is no longer required once malignant transformation has occurred [22]. However, the strong EBV signals detected by the *in situ* hybridization in tumor tissues in the study by Yahia et al. [5] while being absent from the safety margin requires some explanation. The presence of EBV-DNA and an APOBEC mutational signature correlated with adverse clinicopathological features, however, the presence of the virus is not always a requirement for tumor growth, consistent with a "hit and-run" mechanism which would also explain why mining of the TCGA RNAseq data did not show active transcription of EBV [26, 27].

Following EBV infection, the host cell is affected through different mechanisms pertaining to the viral lytic and lysogenic survival strategies. The infection that usually occurs during childhood triggers the immune machinery which attempts to clear the virus, and this may probably be to its own advantage to control the development of another intruder, the tumor. The majority of the asymptomatic carriers harbor up to 50 EBV genomes per million B cells [28]. A virus may trick the host cellular machinery and enter into latency phase. Histone acetylation plays an important role in the switch between the lytic and lysogeny phases by regulating BZLF promoter known as Z. It has been suggested that the balance between recruitment of histone acetyltransferases versus histone deacetylases by transacting factors promotes and decides the switch between latency and lytic reactivation [29]. Viral latency may eventuate in carcinogenesis provided the presence of conducive host (susceptibility factors) and viral (oncogenic latency proteins) exists. During latency the virus successfully evade the host's immune system and persists within the B cells by decreasing its contents to few latent genes [30], six nuclear antigens, three latent membrane proteins and two abundant untranslated RNAs and can persist without being recognized by the immune system and with little interference with the health of the host. Functionally, the oncogenic potential of the virus is associated with its latency molecules such as latent membrane protein-1 (LMP1) [31], but some studies however, reported that this form of the virus (latency) to be associated with good prognosis, while on the other hand its lytic form to be a sign of worse outcome [32]. These, and the authors conclude that this might possibly occur through non-specific anti-tumoral immune response and they consider the virus as a 'double faceted' infectious agent at a time acting as a co-factor for the anti-tumoral immune response. However, this is contradicted by the fact that in patients with good prognosis high frequency of interferon (IFN)-γ and tumor necrosis factor (TNF)-α producing cells were observed, which indicates the existence of a Th1-type polarized immune response in the tumor [32]. Inflammation may also contribute to cancerous and precancerous conditions, mainly through signaling of the highly central and pivotal protein NFkB.

The mechanism which disturbs this perfect host-virus equilibrium which is indicated by the majority asymptomatic carriers is not known yet. It could be inherent in the host or in the virus or in both? EBV usually infects immunosuppressed/immunocompromised individuals [33]. Most of the post-transplant lymphoproliferative disorders (PTLD) which are more common in immunosuppressed transplant patients are EBV-associated [34]. The association with PTLD has been observed particularly following allogenic stem cell transplantation (SCT) [35], which brings the element of the stem cell factor in EBV-associated cancer [36] and may account for receptor promiscuity.

2.2. EBV and APOBEC3 as a genomic modifier

Of the several molecules that confer the spectacularly wide genotypic and phenotypic changes, characteristic of the cancer cell is the APOBEC/AID family of enzymes. Editing by apolipoprotein B editing catalytic subunits proteins 3 (APOBEC3s) is a strong and well-conserved system of the innate immunity that mutates and inactivates viral genomes [37, 38]. These proteins are involved in the system of innate defense against exogenous viruses and endogenous retroelements. EBV genomes in EBV-transformed oligoclonal B-cell lines can be edited by at least one APOBEC3 enzyme [39]. It is possible that APOBEC3 increases the chance of viral DNA integration in the host by inducing mutations and genome instability after viral infection [40].

In an analysis of the TCGA breast cancer data mentioned earlier, EBVness correlated with the presence of the APOBEC mutational signature. Recently, APOBEC3 proteins linked the viral infections to cancer development [41], and now recognized as key players in cancer-associated somatic mutation processes that seem to influence cancer development and progression [42, 43]. In breast cancer, APOBEC3B mRNA was found to be overexpressed in the normal breast epithelial cells transfected with HPV [44], indicating a possible role of APOBEC-mediated mutagenesis in HPV-driven tumor development [45]. APOBEC3G was found to be highly expressed in colorectal tumors and hepatic metastasis, and it has been proposed to promote colorectal cancer hepatic metastasis through miR29 downregulation and consequent derepression of MMP2, a known metastasis activator [46]. Also, it has been involved in microRNA regulation [47].

Both molecules, APOBEC3 [10] and miRNA [Yousif submitted], have been implicated in Sudanese multicase colorectal family, with the striking finding of identify by state of tumor tissues between distant relatives, in contrast to a limited similarity between relatives (identity by decent).

2.3. EBV as a potential epigenome modifier

The relationship between EBV and the epigenetic machinery particularly methylation of CpG moieties is too obvious to oversee. It is embedded in the distant evolutionary relationship of viruses and DNA modification systems of selfish DNA. Several tumors are associated with arthrobacter luteus (Alu) elements in which tumor suppressor genes are more enriched [48] and other markers of selfish DNA including the recently recognized N6-methyladenosine (m6A), the most common internal messenger RNA modification found in eukaryotes and also in RNA of

nuclear-replicating viruses [49]. This modification is catalyzed by an evolutionarily conserved, nuclear, multicomponent enzyme. One of whose subunits, methyltransferase-like 3(METTL3), has been identified and a METTL3 knockout model resulted in an apoptosis phenotype [50]. The infected and EBV transformed cancer cell employs a bundle of these tools including the above, HDAC, methylating enzymes like DNMTA/B to its advantage and survival. Methylome analysis may provide further clues to the contribution of epigenetics to the tumorigenesis process in dictating the function of key cancer genes and genomes.

DNA hypermethylation in cancer genomes usually occurs in the promoter regions of tumor suppressor genes, which can result in silencing of tumor suppressor [51]. In contrast, DNA hypomethylation often targets DNA repeats, which may induce genomic instability and mutation events in cancer genomes [52]. There is evidence that promoter hypomethylation of some genes may be associated with the tumor progression and metastasis of some cancers [53] as well as the initiation of inflammation and immunomodulation [54].The role of DNMT3B in the altered methylation and inactivation of genes in human tumor cells as well as its role in the maintenance of the transformed phenotype is well established. It has significant site selectivity that is distinct from DNMTA1, regulates aberrant gene silencing, and is essential for cancer cell survival [55]. DNMT3A and DNMT3B repress transcription independent of their methylating activities, and this repression is partially dependent upon histone deacetylase activity (HDAC) [56]. DNMT3B-mediated gene suppression may involve both methylation-dependent and methylation-independent HDAC-dependent mechanisms. Histone acetylation, a component of an epigenetic mechanism has a role in the initiation and progression of human cancer as a result of post transcriptional modification [57]. Aberration in HDACs leads to transcriptional repression in genes involved in proliferation, differentiation, invasion, and metastasis [58]. HDAC9 an important factor in mammary carcinogenesis [59] overexpression was associated with higher rates of gene transcription and increased epigenetic marks on the HDAC9 promoter. Methylome of BC is a foundation for metastatic risk "CpG island methylator phenotype (CIMP)" in breast cancer is not yet clearly defined as is in colon cancer, in which it is defined by promoter hypermethylation of at least three of five specific methylation markers [60]. In one study, lobular breast carcinoma was revealed with the highest number of differentially methylated CpG sites indicating its epigenetic unstableness [61]. EBV protein, LMP2A, can cause activation of (DNMT1), which in turn hypermethylate a tumor suppressor gene, PTEN in EBV-associated gastric cancer [62]. DNMT1 over expression mediated by EBV LMP1 and LMP2 and Oncogenic EBV gene, LMP1, can upregulate all of the DNA methyltransferases (DNMTs) [63]. DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines [64], and LMP2a functions in the initiation and progression of cancer by inducing the cancer stem-like cells [24] as aforementioned.

Differential methylation analysis of whole methylome data of breast cancer cases from Sudan provided a possible link between these entities. The results reveal epigenetic dysregulation of major developmental pathways including hippo signaling pathway [Alsiddig, 2015, data online, pending submission], thus providing not only a clue to the stem cell dimension of the disease but also insights to subsequent pathognomonic features of cancer process. It also demonstrated the presence of significant enrichment of EBV-associated pathways with a significant score.

3. TNFα gene methylation

An insightful example of the contribution of the methylation phenotype to breast cancer through modulation of key cancer-related genes is the TNFα. Genetic as well as epigenetic aberrations at the promoter of TNF-α has been reported; its promoter can be methylated with functional modification, and eight DNA variants or "SNPs" have been described within the TNF promoter as reviewed by Bayley et al. [65].

Methylated TNFα promoter and TNFα exon1 were associated with significant suppression of TNF in colorectal tumors [66], although, this has to be reconciled with a contrasting report of TNF-α shown to be highly expressed in breast carcinomas [67]. TNF-α is a multifunctional cytokine that plays important roles in diverse cellular events such as cell survival, proliferation, differentiation, and death. However, when chronically produced and inflammation persists in the tumor microenvironment it may have a critical role in the promotion and progression of cancers by DNA damage, enhancing proangiogenic functions, increasing the expression of matrix metalloproteinases (MMP) and endothelial adhesion molecules and inducing growth-promoting hormones and chemokines that promote tumor development [68]. TNF-α can promote EMT of MCF-7 cells and activates cell migration [69]. This transition generates stem-cellness [70]. Activation of regulatory T cells (Tregs) can cause immunosuppression and has resulted from prolonged exposure to TNF-α [71], which could have a cancer-promoting effect. TNF is hence believed to be a double-edged sword that could be either pro- or antitumorigenic, this double standard phenomenon is also seen in severe infectious diseases such as malaria in which fatal cerebral malaria is associated with high circulating levels of this cytokine [72]. Environmental factors such as malaria exerts selective pressure on the TNF loci and is reflected on common polymorphisms in the human genome like the TNF (–308G/A) in the TNF promoter (–308G/A). This SNP which was found to be associated with protection from malaria [72] was found to be associated with susceptibilities to various types of cancer [73]. This influence on the susceptibility to cancer may be associated with altered TNF production or a neighboring gene in tight-linkage disequilibrium. These reports indirectly suggest that TNF has a tumor-promoting role and that TNF promoter SNPs could be a predictor for cancer risk.

The CD40 ligand (CD40L), a glycoprotein involved in B cell proliferation, antigen presenting cell activation, and member of the TNF receptor ligand family, was reported to confer protection from severe malaria has also significant functional homology with EBV LMP1. In the malaria endemic area of eastern Sudan, elevated levels of CD40L expression were observed in comparison to naive healthy controls from nonmalaria areas.

In an analysis of the methylome of subset of human triple-negative breast cancer the analysis identified significant enrichment in methylation phenotypes of the tumor necrosis factor (TNF) and TNF receptor family (**Table 1**). The attempts to dissect the functionality of the TNF promoter have all concentrated on the genetic aspects of TNF gene regulation, but now with the increasing interest in the epigenetic control of gene regulation and possible significance for disease, it is surprising that little attention has been paid to the possibility that aberrant methylation could play a role in TNF dysregulation.

TNF-α stimulates many signaling pathways by binding to two receptors, TNFR1 (p55) and TNFR2 (p75) [68, 74]. TNFR-1 is ubiquitously expressed, whereas TNFR-2 is mainly expressed in immune cells [75].

Gene symbol	Site of hypermethylation	Site of hypomethylation
TNF	TSS 1500, promoter	–
TNFRSF1A	Body	–
TNFAIP3	Body	–
TNFRSF1B	Body	–
TNFSF11	5′ UTR, promoter, exon	5′ UTR, promoter
TNFRSF10D	Exon, body, promoter	Body
TNFAIP8L1	TSS1500, promoter	–
TNFRSF19	5′ UTR, exon, body, promoter,	–
CIQTNF4	5′ UTR, body, promoter	Exon
C1QTNF5	5′ UTR, body, 3′ UTR, promoter	–
TNFRSF13C	TSS1500, promoter	–
C1QTNF9	TSS1500, promoter	–
TNFRSF13B	TSS1500, promoter	–
TNFRSF11A	TSS1500, promoter	Body
TNFRSF8	Promoter	–
C1QTNF7	Exon	–
TNFAIP8L3	Body	–
C1QTNF1	Body	–
TNFSF12-TNFSF13	Body	–
TNFSF8	–	Body
TNF18	–	Body
C1QTNF6	–	Body
C1QTNF8	–	3′ UTR

Table 1. Differentially methylated TNF and TNF receptor family genes at various CpG sites from Sudanese breast cancer samples, indicating the significant enrichment in methylation phenotypes in this important family of genes and being a target of epigenetic modification in a directed tumorigenesis process.

4. Gene chromosome location and breast cancer

Another key class of molecules identified through this approach is the hypomethylated olfactory receptor genes in Sudanese breast cancer samples. Significant enrichment of differentially hypomethylated olfactory receptor family members were mapped to chromosomes 1 specifically to chr1q44 (*P*-value, 6.867e-20) a cytoband known to be one of the viral integration sites [76]. Moreover, this location is also associated with autoimmune diseases [77] and chronic inflammatory responses induced by physical stimuli from the environment [78]. It seems that the virus selects this environmentally prone site.

According to various studies, chromosome 1 aberration is associated with different cancers, such as neuroblastoma [79], cervical [80], and colorectal [81]. In breast cancer, gains at 1q are found in over 50% of breast tumors [82]. It is reported that the long arm of chromosome1 to be usually associated with karyotypic changes seen in breast cancer and is believed that the development of breast cancer might be caused by inactivation of a gene (s) located on 1q23-32 [83].

5. miRNA as epigenetic actor in breast cancer

microRNA(miRNA) is naturally involved in the biological process across the carcinogenesis from initiation to metastasis and this occurs through the spectrum of genetic and epigenetic mechanisms of the cell. Several miRNA have been reported to be involved in the myriad of the biological processes, for example miR-22 (chromosome 17) can regulate breast cancer stemness and metastasis through a TET-dependent chromatin remodeling [84], and miR-373, miR-520 were found to promote migration and invasion of BC cells.

A differential analysis of the methylome dataset of a Sudanese breast cancer cases and controls identified hypomethylated sites for six different miRNAs, including miR-153-2, miR-2276, miR-30B, miR-1204, miR-141, and miR-300 [Alsiddig, 2015 data on line, pending submission]. Only miR-153-2, miR-2276, and miR30B had been previously associated with breast cancer [85–87]. miR153-2 was of particular interest, since numerous studies linked miR153 to a myriad of epithelial cancers. One study demonstrated that miR-153 upregulation promotes prostate cancer proliferation through downregulation of PTEN tumor suppressor gene [88].A test dataset The Cancer Genome Atlas (TCGA), contained methylation data for 90 samples of healthy individuals and 638 samples of primary tumor. The authors found miR153-2 promoter to be significantly hypomethylated at the exact same CpG sites. Interestingly, another epigenetic regulators, TET2, and TET3 are among the listed targets of miR153-2 as predicted by TargetScan algorithm.

RNA-binding protein sometimes have the same target sequence as miRNA and a notable example is miRNA 29 which competes with ELAVL1 on the same regulatory sites.miRNA29b Stops protein production from other genes that play vital role in metastasis and its isoform are shown to regulate various aspects of the carcinogenesis process in different tumors. However, its target site homology with a key RNA-binding protein like ELAV1/Hur suggest that this micro RNA may play a critical role in the early phase of viral pathogenesis and in coupling of downstream key players like TNFα as shown in **Figure 1**.

6. Conclusion

In this chapter, we review some examples pertinent to questions as of why EBV should be considered a tumor virus, examine molecular evidence for the culpability of EBV as oncogenic virus in relation to the established cases of EBV cancer oncogenesis; the cancer target cell and stem cell, which bring the element of development as an epigenetic reprogramming

process, linking the breast cancer methylome differential methylation to developmental and EBV, dwelling on EBV molecular targets, and how the combined interplay of molecular events in human impinges on pathogenesis and malignancy of breast cancer.

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