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Co-Expressional Protein Products of BRCA-1 and EBV-EBNA-1 Genes in Tissues from Human Female Patients with Breast Cancers: An Immunohistochemical Screening Study

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Abstract : Back ground: Breast malignancies are most frequently diagnosed among women in many populations world-wide as well as in Iraq. Although the genetic mutations in BRCA -1 and BRCA - 2 genes are still constituting up to 90% of the total risk for breast cancers, yet many indirect evidences are supporting a role for an association of EBV with such cancers.
Objective: To analyze the rate of EBV infection in the breast tissues in association with defects and / or mutations in BRCA-1 gene, by assessing the endogenous levels of the total expressed BRCA-1- as well as EBNA-1 protein products, and their relations to the differentiation of primary invasive breast cancer tissues.
Patients and methods: Fifty-four (54) formalin-fixed, paraffin- embedded breast tissues were obtained in this study; (34) biopsies from breast cancers (BC) and (20) from apparently normal breast autopsies as a control group. Detection of protein expressional products of EBNA-1 gene of Epstein Barr Virus as well as BRCA-1gene was done by HRP/DAB immune-enzymatic antigen detection system using specific rabbit-anti-human primary antibodies for EBV-EBNA -1as well as defected or mutated BRCA-1 protein products.
Results: Detection of EBNA-1 - immunohistochemical (IHC) reactions in tissues with BC was observed in 12 out of 34 (35.3%), while in healthy breast tissues in the control group was detected in 10% (2 out of 20). Detection of BRCA-1- protein- immunohistochemical (IHC) reactions in tissues with BC was observed in 16 out of 34 (47.1%), while none of the examined healthy breast tissues in the control group revealed such IHC-reactions. The difference between the percentages of BRCA-1- as well as EBNA1 proteins detection in BC tissues & control group was statistically significant (<0.05). Among breast cancer tissues that showed score I of IHC reactions for BRCA1, 68.8% have well differentiated grade; and 18.7% of those tissues that have score II-IHC reactions showed moderate differentiated grade and lastly, 12.5% of the BC tissues which showed score III have presented as poorly differentiated BC tissues. However, statistical significant differences between the frequencies of EBV-EBNA1 and BRCA-1- immunohistochemical reactions were neither observed in relation to the age of these breast cancer patients nor to the grade of invasive breast cancer tissues (P value > 0.05).
Conclusions: Our results indicate that the EBV might contribute to the development of subset of breast tumors. The present results of the rates of defects or mutations in the BRCA-1- genes in relation to the grade of breast cancer tissues also could point for their occurrence and contribution as early events in breast carcinogenesis.
Keywords : Breast cancer; Epstein Barr Virus; EBV - EBNA 1;Defects / Mutations; BRCA-1- Gene; Immunohistochemical technique.

Introduction:

Globally, breast cancers are the most frequent malignancies that affect women[1-3].In Iraq, Iraqi Cancer Board as well as Iraqi Cancer Registry Center in Iraqi Ministry of Health has recently demonstrated that female breast cancers constituted about 25% out of the total registry of cancers in Iraqi patients[4].

A variety of reproductive and hormonal factors have been identified in the etiology of breast cancer constituting together fifty percent among all the etiologies of breast carcinogenesis and as such, the research works were prompted to unravel other risk factors in that critical issue[5].

Genetic factors were noticed to play a recognized role in less than 5% of breast cancer leading to hereditary breast- ovarian cancer syndrome[6]. However, and on exclusion the risk of the familial history, the risk of breast carcinogenesis is significantly increased in relation to the occurrence of some mutations, particularly in BRCA-1 and BRCA-2 genes[7].

Among human tumor suppressor genes, the BRCA-1 and BRCA-2 genes are ubiquitously found in all humans where their genes and proteins are called breast cancer type 1 and 2 susceptibility genes and proteins, respectively[8].

Certain variations of the *BRCA1* gene lead to an increased risk for breast cancer as part of a hereditary breast - ovarian cancer syndrome. Researchers have identified hundreds of mutations in the *BRCA1* gene, many of which associated with an increased risk of cancer. Women with an abnormal BRCA1 or BRCA2 gene have up to an 80% risk of developing breast cancer by age 90; increased risk of developing ovarian cancer is about 55% for women with BRCA1 mutations[9].

Approximately 50% to 65% of women who have a deleterious mutation in *BRCA1* could develop breast cancer by age 70, and 35% to 46% could develop ovarian cancer by age 70. Approximately 40% to 57% of women with a deleterious mutation in *BRCA2* will develop breast cancer by age 70, and 13% to 23% will develop ovarian cancer by age 70(10).

Viruses are involved in the development of various cancers[11]. In 1995, the Epstein-Barr virus (EBV), and ubiquitous herpes virus, was found in 21% of 91 breast cancers[12].

Since then, a large number of studies have detected EBV infection in patients with breast carcinoma. A series of studies that adopted non-breast-cancer control groups have also been performed[12,13],and several mechanisms and hypotheses about the association between EBV infection and breast carcinoma have been developed [11, 14,15].

Some researchers believed that EBV infection may play a role in the early stages of breast carcinogenesis and elevate breast cancer risk[16].Moreover, EBV infection might be a latent factor in the development of certain types of breast carcinoma (15). However, statistical data from studies have varied widely. This inconsistency could be largely attributable to several problems: technical challenges in detecting and localizing the EBV in tumor cells, study designs that involved a specific histological type of breast carcinoma, and the lack of an epidemiological perspective that could clarify the inconsistencies in EBV prevalence across studies[17].

Epstein-Barr virus (EBV) is a large double-stranded DNA virus that is classified as a gamma-1 herpes virus of the lymphocrypto- genus.EBV has infected greater than 90% of the world's population and is the etiologic agent of infectious mononucleosis[18].

In the growth program of EBV expresses all the nine known latent proteins: the six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNALP), three latent membrane proteins (LMP1, LMP2A, and LMP2B), and the non-polyadenylated EBV RNAs (EBERs)[19].

In the default program, EBNA1, LMP1, LMP2A, and the EBERs are expressed, providing necessary signals that are thought to allow infected lymphoblasts to differentiate into memory B cells[290]. In the latency program, which has a much more restricted pattern of viral gene expression, very few viral genes are expressed. The transcript for LMP2A has been consistently detected, and recent reports suggest the EBERs are also expressed.This low level of viral gene expression allows persistence of the virus in resting recirculating memory cells in a way that is nonpathogenic and not detectable by the immune system[21].

Production of infectious virus is the essential feature of fourth and final gene expression program found in humans latently infected with EBV[20].

This study was proposed to unravel the rates of both EBV infection as well as defects and / or mutations in BRCA-1 gene in the breast tissues and their relations to the differentiation grades of primary invasive breast cancer tissues.

Materials and Methods:

Study Groups:

This study was designed as a retrospective research; a number of (54) formalin-fixed, paraffin embedded breast tissue blocks enrolled in this study which comprised both patients and control samples that their age ranged from 32 to 71 years. These retrospective paraffin-embedded samples were retrieved from the archives of the period from 2011 till 2016 belonging to major hospitals and private histopathological laboratories in Kerbela, Babylon, and Al-Najaf provinces. The diagnoses were based on their accompanied pathological reports of the corresponding patients. These blocks included a group of (34) biopsies from patients who had undergone surgical operation or biopsies for their breast cancers (BC) and (20) autopsies from apparently normal breast tissues control group. These breast tissues were properly subjected to fixation as well as paraffin embedding and used for this research work as an age- and grade- matched groups.

Laboratory methods:

Slide Preparation:

Tissue sectioning was conducted following trimming process of the tissue blocks at the histopathological department of Teaching laboratories / Al- Sadar Medical City (Al- Najf)&Al_Hilla hospital teaching and a second confirmatory histopathological re –evaluation of each obtained tissue blocks was done by a consultant pathologist. One paraffin embedded (4 mm) thick-tissue section was prepared and mounted on ordinary glass slide and stained with hematoxyline and eosin, while other (4 mm) thick-tissue sections were stuck onto positively charged slide to be used for detection of EBV-EBNA 1& BRCA1 – antigen using Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (Lot. Number: ab64264) that was purchased from (Abcam, UK) , an immunoenzymatic antigen detection system for immunohistochemistry techniques, using specificRabbit Monoclonal primary Anti-EBV Nuclear Antigen antibody [E1-2.5] (ab8329) [Lot. Number: [E1-2.5] ab8329], was also purchased from (Abcam, UK) andBRCA1[Lot. Number:(ab191042)], also purchased from (Abcam, UK) .The details of methods for performing IHC reaction with these antibodies were conducted according the instructions of that manufacturing company, and were done in the Research Laboratories of the Clinical Communicable Diseases Research Unit, at College of Medicine, University of Baghdad as well as in the Advanced Microbiology Research Laboratory at College of Science, University of Babylon.

Histopathological Analysis:

According to the specification of the kit, proper use of this IHC detection system gives an intense brown signal at specific sites of the expression protein in positive test tissues (by using light microscope).

The signal was evaluated under light microscopy using × 100 lens for counting the positive cells. The IHC results were given intensity and percentage scores based on intensity of positive signals and number of cells that gave these signals, respectively.

Positive cells were counted in 10 different fields of 100 cells for each sample and the average percentage of positive cells within the 10 fields was determined. A scale of 0-3 was used for relative intensity with 0 corresponding to no detectable IHC reaction, and 1, 2, 3 equivalents to low, moderate, and high intensity of reaction respectively. Cases were assigned to one of the following percentage score categories: 1%–25% (score 1), 26%–50% (score 2) or > 50% (score 3)[22].

Statistical Analysis:

T test, ANOVA test, and Chi square were applied for statistical examination of results obtained in our research. All these statistical analysis were done by using Pentium-4 computer through the SPSS program (version-19) and Excel application.

Results

Archival specimens enrolled in this study were related to female patients with breast cancer whom mean age was (52.7+ 8.5 years) while the mean age of those who have apparently healthy tissues was (62.7 + 7.2 years) (Table 1).

Table (1): Studied groups according to the mean age

Study Groups	N	Mean (years)	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Patients	34	52.7	8.5	1.5	49.5	55.9	38.00	69.00
Control	20	62.7	7.2	2.2	58.1	67.3	54.00	76.00
Total	54	55.6	9.3	1.5	52.7	58.4	38.00	76.00

		t-test for Equality of Means						
		Independent Samples Test:					95% Confidence Interval of the Difference	
		t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Age		-3.579	40	.001	-10.00000	2.79444	-15.64777	-4.35223

Thesignals of EBV-EBNA1 1 immunohistochemical (IHC) reactions were detected as brown discoloration at the antigenic sites that were detected by their specific primary antibodies (Figure 1). Table (2)shows the positive results of EBNA 1 - IHC reactions, where 35.3% (12 of total 34) breast cancers showed positive signals while 2 out of 20 (10%) in control group has presented such positive signals for IHC test. While, the highest percentage of EBNA 1 - IHC score signaling (50%: 6 out of 12 cases) was found in the moderate score (score II), whereas 41.7% (5 out of 12 cases) and 8.3% (1 out of 12cases) were found within low (score I) and strong (score III) scores, respectively. Statistically, significant differences ($p<0.05$) were found on comparing the percentage of EBNA1 in the BC group according to their positive signal scoring.

Table (2): Signal scoring of EBNA 1- immunohistochemical reactions among the breast cancers tissues.

P	Normal Breast Tissues (n=20)		Malignant Breast Tumors (n=34)		EBNA-1 Signal scoring	
	%	N	%	N		
0.001 significant	80	18/20	64.7	22/34	**Negative**	
	10	2/20	35.3	12/34	**Positive**	
	100	2	41.7	5/12	**I**	**Scoring**
	0	0	50	6/12	**II**	
	0	0	8.3	1/12	**III**	

The highest percentage of EBNA 1 signal intensity in the present study was (41.7%: 5 out of 12 cases) that is related for each weak and moderate intensity, while 16.6% (2 out of 12) of the breast cancers tissues were presented with strong intensity . Significant statistical differences ($p<0.05$) were found among breast cancers tissues according to their EBNA 1- scoring intensities (Table3).

Table (3): Signal Intensities of Positive EBNA1- IHC Reactions.

Chi-square Tests	Negative EBNA1 signaling	Signal Intensity			positive EBNA1 signaling	Studied Groups
		High	Moderate	Weak		
<0.001 significant	22/34 (64.7%)	2/12 (16.6%)	5/12 (41.7%)	5/12 (41.7%)	12/34 (35.3%)	**Malignant Br. Tumors (n=60)**
	18/20 (80%)	0/2 (0.00%)	1/2 (50%)	1/2 (50%)	2/20 (10%)	**Healthy Br. Tissues (n=20)**

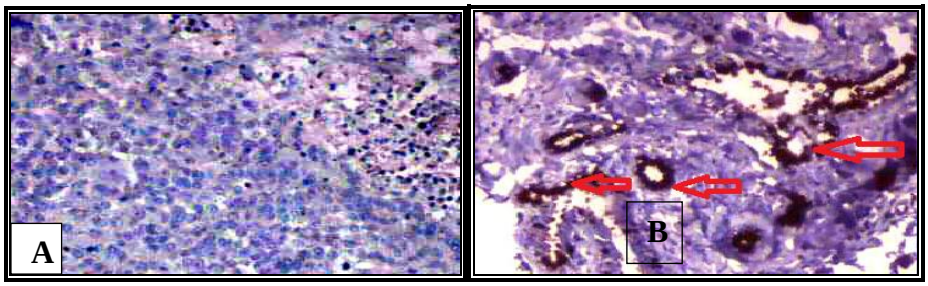

Figure (1): Infiltrative Breast Carcinoma Showing The Results of Immunohistochemistry Staining Protein Over Expression Using Biotinylated Anti-EBNA1 Protein Antibody; Stained By DAB-Chromogen (Brown) and Counter Stained By Mayer's Heamatoxylin (Blue).

A- Breast Cancer with negative staining with EBNA1 .

B-EBNA1-IHC-reaction with high signal score and strong signal intensity (4x)

It was noticed that 12 cases out of 34 (35.3%) of EBNA1-IHC test showed positive EBNA1-IHC reactions .It was found that 66.7% of breast cancer tissues that showed score I of IHC reaction for EBNA1 have well differentiation; and 75% of breast tissues that have score II of IHC reaction for EBNA1 have moderate differentiation. Lastly, 60% of the BC tissues cases that showed score III have presented with poor differentiation. Statistically, the overall EBNA1 –IHC scoring on comparing to tumor grading showed non- significant differences (P>0.05) Table (4) .

Table (4): The correlation of EBNA1-IHC score with grading of breast carcinoma

P	Breast Cancer Grades						EBNA1 score	
	Poorly differentiated (n=15)		Moderately differentiated (n=11)		Well differentiated (n=8)			
	%	N	%	N	%	N		
0.1[N.S]	66.7	10/15	63.6	7/11	62.5	5/8	**Negative**	
	33.3	5/15	36.4	4/11	37.5	3/8	**Positive**	
	20	1/5	25	1/4	66.7	2/8	**I**	**Scoring**
	20	1/5	75	3/4	33.3	1/8	**II**	
	60	3/5	0.00	0/4	25.0	0/8	**III**	

The signaling results of immunohistochemical reactions for BRCA-1antigenic detection were observed as brown discoloration at the specific antigenic sites of these reactions with their specific primary antibodies Figure (2).

The positive- signal results of BRCA-1 - immunohistochemical reactions were found in 47.1% % (16 out of total 34)breast cancers while no tissue in the control group has showed such IHC signals. The statistical Pearson Chi-Square analysis shows significant difference between the patients and control groups regarding BRCA-1 immunohistochemical results (<0.05) Table (5).

Table (5): Frequency of BRCA-1 immunohistochemical reactions among the study groups

				GROUP		Total	Valid Percent	Cumulative Percent
The Marker				Patients	Control			
BRCA-1		Positive	Count	16	0	16		
			%	47.1%	0.0%	33.3%	47.1	47.1
		Negative	Count	18	20	38		
			%	52.9%	100.0%	66.7%	52.9	100.0
	Total		Count	34	20	54		
			%	100.0%	100.0%	100.0%	100.0	

		Value	df	Asymp. Sig. (2-sided)
BRCA-1	Pearson Chi-Square	8.400	1	.004

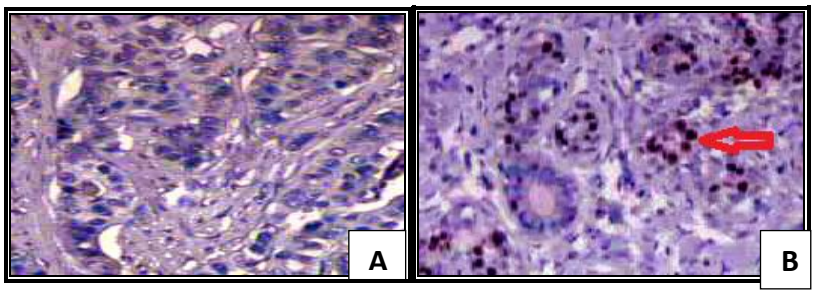

Figure (2): Infiltrative Breast Carcinoma Showing The Results of Immunohistochemistry Staining Protein Over Expression Using Biotinylated Anti-BRCA1 Protein Antibody; Stained By DAB-Chromogen (Brown) and Counter Stained By Mayer's Heamatoxylin (Blue).

A- Breast Cancer with negative staining with BRCA1 .

B-BRCA1-IHC-reaction with moderate signal score and strong signal intensity (4x)

In the present study, 16 tissues out of 34 showed positive EBV-EBNA 1 -IHC reactions .It was found that 62.5% of breast cancer tissues that showed score I of IHC reaction for EBNA- 1 have well differentiation; and 63.6% of breast tissues that have score II of IHC reaction for EBNA 1 have moderate differentiation. Lastly, 26.7% of the BC tissues cases that showed score III have presented with poor differentiation. Statistically, the overall EBNA 1– IHC scoring on comparing to tumor grading showed non- significant differences (p>0.05) Table (6).

Table (6):Correlation of EBNA 1 -IHC scoring with the grading of breast cancers .

P	Breast Cancer Grading						State Of Signal Scoring	
	Poorly differentiated (n=15)		Moderately differentiated (n=11)		Well differentiated (n=8)			
	%	N	%	N	%	N		
0.54 [N.S]	73.3	11/15	36.4	4/11	37.5	3/8	Negative	
	26.7	4/15	63.6	7/11	62.5	5/8	Positive	
	25	1/4	71.4	5/7	20	1/5	I	Scoring
	25	1/ 4	14.3	1/7	80	4/5	II	
	50	2/ 4	14.3	1/7	0.00	0/5	III	

Discussion

Breast cancers have ranked the top of the commonest ten cancers in the Iraqi provinces and districts, accounting for about one third of the registered female cancers[23].The role of EBV in breast cancer etiology is still controversial. Unraveling the relationship of EBV in such cancer is potentially important as to a better understanding of breast carcinogenesis as well as for early detection and the prevention of such cancer[24].

Reviewing the 34 breast cancers patients included in this study, it was found that their age was ranging between 38-69 years and their mean age was 52.8 + 8.6 years. These results are consistent with those Iraqi as well as world-wide reported results which have found that breast malignant tumors are usually affecting females aged over forty years[25-27].

Generally, aging is a risk factor that increase the possibility of malignant changes noticed in breast epithelial tissues which was found to increase with age. The present data could also point for the importance of such risk factor in the tumorigenesis of the studied BC tissues and might be related to the effect of long exposure of these breast epithelial tissues to the hormonal changes[28].

In the current study ,the results have shown EBV-EBNA1 – IHC reactions positivity in 35.3% (12 out of 34 cases) of those breast tissues obtained from a randomly collected Iraqi patients with breast cancers , while it was detected in only 2 out of 20 (10%) of breast tissues in the control group. The importance of such findings was their relevance and in line with many observations which are indirectly supporting an association of EBV with breast cancer : (a) EBV is detected in breast milk of some women[29] transfection of EBV DNA stimulates growth of human breast milk cells[30]; (c) some EBV-associated lymphomas have occurred in breast[31,32]; (d) epidemiological similarities of breast cancer to young-adult Hodgkin'slymphoma[33]; (e) EBV detection in benign breast tumors in immunosuppressed women[34]; (f) EBV-lymphoblastoid cell lines can directly infect breast epithelial cells on contacting them and (g) serological evidence of anti-EBNA-1 antibodies in stored sera of breast cancer Indian women[35].

IARC Working Group has classified EBV as a group-1 carcinogen[36]. However, the associated cancers vary markedly in their viral prevalence (nearly 100% in nasopharyngeal carcinoma to about 10% in gastric carcinomas)[37,38].

The patterns of viral genes expression also differ, suggesting that EBV may affect cell growth in more than one way[39].Although in the current study the detected EBV infection could represent an important step in carcinogenesis, yet is not a sufficient one , and as such we believe that an additional epidemiological risk factors, and stated by Sally L. Glaser*et al.*[40], could have played a critical role in this process.However, our results are comparable to the results reported by Zekri*et al.*,[24] where PCR for EBNA1 has detected EBV infection in 23 (57.5%) out of those 40 studied Egyptian cases and in 16 (32%) out of those 50 studied Iraqi cases with invasive breast carcinoma.

The current finding is in close to the previous study done by Joshi et al.[41]where they found that about 55% of breast cancer Indian women cases showed EBNA-1 expression in tumor cells by IHC, while all the controls

with benign breast disease were negative. The results of the current study are also compatible with and / or consistent to the findings of numerous studies, as those done by Chu et al.[42]: 25%, Murray et al.[43]: 31%, Preciado[44]: 38/102 (37%), Fawzy*et al*.,[45]: 10/40(25%), Lorenzetti et al.[46]: 22/71(31%) of EBNA-1 expression in breast cancer cells by IHC.

However, the present findings are lower than a study done by Bonnet *et al*.,[47] where they found that 9/9 (100%)of EBNA-1 expression in breast cancer cells by PCR. In addition, The obtained results in the present study are in disagreement with the study done by Brink *et al*.,[48] who found that about 2% (2 out of 115 cases) EBNA-1 expression in tumor cells by IHC.

Lin *et al*.,[49] demonstrated that EBV infection promotes tumorigenic activity of breast cancer cells. It is suggested entry of EBV into epithelial cells involves an epithelial-specific receptor or cell–cell contact with infected lymphocytes .

In the present study, the results have shown BRCA-1- protein- IHC reactions positivity in 16 out of 34 (47.1%), while none of the examined healthy breast tissues in the control group revealed such IHC- reactions.The BRCA-1 gene spans approximately 80 kb of genomic DNA region make it relatively unstable and prone to deletions and rearrangements (50).Indeed, 36% of mutations in Dutch families are due to three different, large genomic deletions in BRCA-1(51), and duplication of exon 13 has been reported in a BCLC family linked to BRCA1.

Several studies have compared survival of BRCA1-associated breast cancer to that of BRCA1-negative cases with conflicting results[52,53].

However, the pathological features of BRCA1-associated tumors would predict a poorer survival in this group of patients.The mutation prevalence estimates may also be biased by preferential survival between cases with mutations and those without. For many oncologists it is not yet possible to draw evidence-based conclusions about the association between *BRCA1* and/or *BRCA2* mutation carrier ship and breast cancer prognosis, relating that to the heterogeneity of the reported results which precluded a conclusion regarding the contribution of *BRCA1/2* status and tumor features to a worse survival. However, primary breast cancer treatments may be different for *BRCA1* and *BRCA2* mutation carriers compared to non-carriers, mostly related to different pathological features of tumors in carriers (S10 Supporting Information, part A)[54,55].In addition, therapy response of tumors in *BRCA1/2* mutation carriers might be better compared to that in non-carriers[56].

In the current study, it was found 68.8% of breast cancer tissues that revealed BRCA1 mutation have well grades, followed by moderate grade (18.7%) and poor grade (12.5%).In this study, the percentage of BRCA1 was found to decrease with the proceeding of the grading of breast cancer. Their BRCA1-negative BC counterparts tissues were found to have a similar decreasing trend of grades of BC.

The grade of BRCA1-related breast cancers has been shown to be consistently elevated with grade 3 comprising 61.3–81.5% of cases versus 22–27% of hospital-based comparison series[57, 58]. In this report, 65% of tumors from BRCA1 patients were high grade. Although a higher proportion of grade 3 tumors has been reported for BRCA1- related breast cancers[57, 58], survival has been reported as comparable or better[58,59]. Additionally, hereditary breast cancer presents at a slightly younger age[60], and a young age has been demonstrated to be an independent adverse risk factor[60-63]. Thus, grade 3 in BRCA1-related breast cancer may have a different implication for prognosis than in other breast cancer cases[64].

According Bordeleau and colleagues review[65], the overall prognosis of *BRCA*-associated breast cancer was similar to that of breast cancer not associated with *BRCA* mutations. For studies published in the 1990s, several methodological limitations have led to an inconclusive results whereas for more recently published studies, with an improved methodology, still failed to demonstrate a significant overall survival difference. In 2010, Lee and colleagues[66], found that *BRCA-1* mutation carriers had significantly lower short-term and long-term overall survival rates (OSR) relative to non-carriers while both short-term and long-term OSR of *BRCA2* carriers did not differ from non-carriers.

On the basis of systematic and evidence-based analysis of all studies published to date, to explain the large heterogeneity between the reported results, surprisingly, only two factors seem to explain part of the heterogeneity; misclassification bias; when a study had not tested the comparison ('non-carriers') group, and the

proportion of incident cases (S11 Supporting Information, panels C and D). Other reasons for the large heterogeneity and generally weak associations observed might be population differences (i.e. different mutations), differences in completeness of follow-up (often not reported), differences in consideration of contralateral breast cancer and prophylactic surgeries (usually not reported). Publication bias is unlikely to play a large role, because of the low prevalence of *BRCA1/2* mutations in populations as well as included published studies with only a small number of carriers[67].

From our results we can conclude that EBV might contribute to the development of subset of breast tumors. The present results of the rates of defects or mutations in the BRCA-1- genes in relation to the grade of breast cancer tissues also could point for their occurrence and contribution as early events in breast carcinogenesis.

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