RESEARCH ARTICLE

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Prevalence of Hepatitis B Virus Markers among the Women with Breast Cancer

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Abstract

Introduction: Breast cancer represents a formidable peril to the female populace on a worldwide level. The association between breast cancer and various factors, including viral infections, has been extensively investigated. Recently, the link between HBV infection and breast cancer patients has garnered attention. The present research aims to assess the prevalence of HBV markers among women diagnosed with breast cancer in Ahvaz city, Iran. **Materials and Methods:** Serum specimens were procured from 90 patients who had been clinically diagnosed with breast cancer. The age of the patients ranged from 29 to 80 years, with a mean age of 49.42±10.7. Histological examination of biopsy specimens revealed that 75 (83.33%) were ductal, 11 (8.88%) lobular, 2 (2.22%) mucinous, 1 (1.11%) medullary, and 1 (1.11%) was metastatic. The serum samples were subjected to initial HBsAg and anti-HBc testing via ELISA. Samples that tested seropositive (HBsAg + anti-HBc) were subsequently analyzed for the S region of HBV through nested PCR and DNA sequencing. Finally, a phylogenetic tree was constructed for positive HBV DNA tests. **Results:** Among the 5/90 (5.55%) cancer patients, it was found that 3 (3.33%) cases of ductal carcinoma and one (1.11%) lobular carcinoma displayed positivity for HBV markers (HBsAg, anti-HBc, HBV PCR). Notably, one (1.11%) patient with ductal carcinoma solely demonstrated anti-HBc positivity. The phylogenetic tree analysis of the S region revealed that all HBV strains identified were categorized as genotype D. **Conclusion:** The statistical analysis did not reveal any significant findings (p= 0.315) in the distribution of cancer types across different age groups. Among patients diagnosed with breast cancer, a notable prevalence of 5.5% was observed in HBV markers. The dominant HBV genotype among breast cancer patients was identified as genotype D.

Keywords: Hepatitis B Virus- carcinoma- ductal- breast- nested polymerase chain reaction

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Introduction

The chronic infection of HBV, which affects approximately 257 million individuals worldwide and results in 887,000 annual deaths [1], has been reported to be accountable for acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma worldwide [2-5]. Rezaei et al. [6] recorded a reduction in the occurrence of hepatitis B infection in Iran, whereby the frequency dwindled from 3.02% in 2000 to 1.09% in 2016. Furthermore, it was noted that the prevalence of Hepatitis B was more than 1.3 times higher in males than in females in 2016.

Breast cancer can be a leading cause of death in women worldwide. It is estimated that there are approximately 2.3 million new cases of breast cancer and 685,000 deaths worldwide each year [7]. It is expected that one in nine women will develop breast cancer and one in 29 will die from the disease [8]. Several factors may be associated with developing breast cancer, including getting older, genetic mutations in certain genes (BRCA1 and BRCA2), family history of breast or ovarian cancer, chest radiotherapy, exposure to diethylstilbestrol (DES), excessive alcohol consumption, significant overweight apostrophe hormone replacement therapy (HRT) after menopause [9-12].

The primary classifications of breast cancer consist of non-invasive (or in situ) or ductal carcinoma in situ (DCIS), invasive, and metastatic breast cancer [13, 14]. Invasive ductal carcinoma (IDC) represents approximately 80% of all breast cancers and is the most frequently occurring subtype (IDC). Invasive lobular carcinoma, or ILC, represents the second most prevalent form of breast

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cancer, accounting for 10 to 15% of all breast cancer cases. While ILC can affect women of any age, it typically manifests in older women. [13-15]. Metastatic breast cancer, also referred to as advanced or stage IV breast cancer, denotes a late-stage condition whereby the disease has spread to other organs in the body, including the lymph nodes, lungs, liver, bones, and brains [10, 13, 14].

According to Bonhoeffer et al. [16], Hepatitis B virus falls under the classification of Hepadnaviridae. Recently, HBV has reclassified into 10 genotypes (A-J) based on more than 8% genetic variability [17]. It has estimated the nucleotide substitution rate of HBV to be about $1.4 - 5.0 \times 10^{-5}$ per site per year [18].

Chronic Hepatitis B Virus (HBV) infection results in persistent harm to hepatocytes, thereby resulting in the impairment of estrogen inactivation and the circulation of elevated levels of unbound estrogen in the bloodstream, which has the potential to stimulate the development of breast cancer [19].

Occult hepatitis B infection (OBI) represents a condition characterized by the absence of detectable hepatitis B surface antigen (HBsAg) in the serum, while still exhibiting detectable levels of hepatitis B virus (HBV) DNA in both serum and/or intrahepatic compartments. The presence of mutations within the preS1, preS2, and S regions of the HBsAg gene can lead to the absence of detectable HBsAg [20].

Iran is considered to be an area with a low prevalence of HBV, with an estimated rate of HBsAg carriers being 2.2% [21]. In 2019, the coverage of 3 doses of the HBV vaccine reached 85% worldwide, compared to around 30% in 2000, resulting in a significant reduction in HBV carrier rates and hepatitis B-related morbidity and mortality [22].

The majority of cancer patients with asymptomatic hepatitis B virus are not conscious of their infection. Reactivation of HBV has been documented in breast cancer patients receiving chemotherapy [23, 24]. The outcome of HBV reactivation is acute fulminant hepatitis, liver failure, and death [25, 26]. Therefore, the aim of this study was to investigate the occurrence of HBV in breast cancer patients in Ahvaz city, capital of Khozestan, Iran.

Statistical analysis

Data analyses were conducted utilizing the Statistical Package for the Social Sciences 22.0 (SPSS Inc., Chicago, IL, USA). The Chi-Square test was employed for computation purposes. The acceptance of a significance level of $P<0.05$ was established.

Materials and Methods

Ninety female patients diagnosed with breast cancer were the subjects of this study. Serum samples were collected from Glestan and Apadana hospitals in Ahvaz cities during Feb 2020- Jan 2022. The study encompassed certain parameters for the inclusion criteria, namely the females who underwent surgical intervention within a hospital setting. Conversely, the exclusion criteria took into account patients with bilateral breast cancer and those afflicted with metastatic cancer.

ELISA screening was implemented on all serum specimens in order to identify the presence of HBsAg and anti-HBc. Serum specimens that exhibited positivity for both HBsAg and anti-HBc, as well as those that solely displayed positivity for anti-HBc, or demonstrated negativity for both HBsAg and anti-HBc, were subjected to nested PCR for the purpose of amplifying the partial S region of HBV DNA. The DNA that underwent amplification was subsequently subjected to sequencing. The findings of the breast cancer biopsies were subsequently verified by a pathologist.

Molecular testing

DNA was isolated from the serum of patients using a DNA extraction kit (Sinaclon, Iran) in accordance with the manufacturer's instructions. The extracted DNA was stored at -20°C until further analysis was required.

Nested PCR

For the first round, the following outer primers of the S region, S1-F: 5′-CATCAGGATTCCTAGGACCCCT-3′ and S2-F: 5′-CTTGTTGACAAGAATCCTCACA-3′ were used PCR test [27]. The reaction mixture utilized in the PCR process contained 5 µl of an isolated DNA sample, which was quantified at 400 ng and served as the template. Additionally, the mixture contained 2.5 µl of a 10x reaction buffer, 0.75 µl of MgCl2 (50 mM), 0.5 µl of forward/reverse primer, 1 µl of dNTP (10 mM), 0.2 Cinna Gen Taq DNA Polymerase (5 u/µl), and double-distilled water up to 25 µl. The thermal cycler (TC-512, Techne, UK) was utilized for the reaction mixture, which underwent an initial denaturation at 94°C for 5 minutes, followed by 40 cycles at 94°C for 45°C, 52°C for 45s, 72°C for 45s, and a final extension at 72°C for 8 minutes. For the second round, the following inner primers were implemented, S3-R: 5′-AGGACAAACGGGCAACATAC-3′ and S4-R: 5′-CCAACAAGAAGATGAGGCATA-3′ [27].

For the second round, 2.5 µl of the PCR product derived from the initial round was introduced to a PCR reaction mixture containing identical components as those stipulated for the first round and then subjected to a thermal cycler. The thermal cycler was programmed in the following sequence: initial denaturation at 94°C for 5 minutes, followed by 40 cycles at 94°C for 45°C, 52°C for 45s, 72°C for 45s, and a final extension at 72°C for 8 minutes. All reactions were conducted in the presence of both negative and positive controls.

Sequencing and phylogenetic analyses

The amplified products obtained from PCR that displayed affirmative outcomes were subjected to sequencing in both the forward and reverse directions. This sequencing process was conducted using the Applied Biosystem 3500 instrument, manufactured by ABI Scientific in the United States. Subsequently, the nucleotide sequencing analysis of the isolated partial "S" region of the Hepatitis B Virus (HBV) was aligned with the HBV database available on the National Center for Biotechnology Information (NCBI) website. This alignment was performed to determine the genotype of the HBV. The results obtained from the partial sequencing of the "S" region were then submitted to GenBank with the

purpose of acquiring an accession number. To construct the phylogenetic tree for the isolates of the partial "S" region, the Neighbor Joining method was employed. This method utilized the Kimura 2-parameter distance model and was executed with 1,000 bootstrap replicates. The analysis of these methods was carried out using the MEGA software version 6.

Results

The age of the patients ranged from 29 to 80 years, with a mean age of 49.42±10.7 years.

The occurrence of breast cancer among a diverse range of ages exhibits a minimum occurrence of 1.11% in individuals under the age of 29, while the highest incidence of 27.77% is observed in the 40-49 age category for ductal carcinoma. Additionally, the prevalence of lobular carcinoma varies from 1.11% in individuals over the age of 80 to 5.55% in those belonging to the 50-59 age group. However, these findings did not yield statistically significant results (p=0.315) (Table 1).

The GenBank database contains the recorded sequences of four partial S regions, which are identified by the accession numbers OR021896-OR021899. Through the process of sequencing and blasting, it was discovered that all four isolates of the HBV virus belong to HBV genotype D.

The patients who had various forms of cancer and different stages of cancer displayed three cases (3.33%) of ductal carcinoma, with one case (histological grade III) and two cases (histological grade II). Additionally, one case (1.11%) of lobular carcinoma (histological grade II). All of these cases exhibited positive HBV markers, including HBsAg, anti-HBc, and HBV PCR. Interestingly, there was a single patient (1.11%) with ductal carcinoma of histological grade II who exclusively demonstrated anti-HBc positivity, as shown in Table 2.

The phylogenetic tree analysis

The findings of the phylogenetic tree analysis of the S region of HBV isolates unveiled that all four HBV isolates (OR021896-OR021899) obtained from Ahvaz city, which are denoted by black circles, are grouped together with genotype D2 from Russia (OP256019) and genotype D from Iran (JN040804). This is illustrated in Figure 1.

Discussion

Approximately 15% of human malignancies can be attributed to viral infection, with viruses being the second most substantial risk factor for cancer following tobacco [28]. The involvement of various viral pathogens, including the hepatitis C virus, human papillomavirus, cytomegalovirus, Epstein Barr virus, BK polyomavirus, JC polyomavirus, and Merkel cell polyomavirus (MCV), have been thoroughly documented with regard to the incidence of breast cancer in the female demographic [29-34].

The examination of the phylogenetic relationships demonstrated that all four HBV isolates from patients with breast cancer pertain to genotype D, which represents the dominant genotype prevalent in Iran [35].

The protracted persistence of HBV infection may ultimately result in the emergence of breast cancer. Recent research has documented the expression of the HBV X protein (HBX) in breast tissue, which acts to inhibit the p53 tumor suppressor gene, thereby encouraging the carcinogenesis of breast cancer [36]. Various studies indicate that hepatitis B X-interacting protein can activate a range of viral and cellular promoters. Furthermore, high expression of HBXIP has been observed in breast cancer cells [37-41]. HBXIP additionally initiates the activation of the transcriptional coregulatory protein, LIM-only protein 4 (LMO4), via the transcription factor Sp1, thereby facilitating the proliferation of breast cancer (BC) cells [40]. Moreover, it augments the process of angiogenesis and the growth of BC cells by modulating fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) [42]. Furthermore, it intensifies the growth and migration of BC cells through S100A4 both in vivo and in vitro [37], while concurrently upregulating Lin28B expression, thus exerting a promoting effect on BC [39].

In our investigation, we observed a general positivity

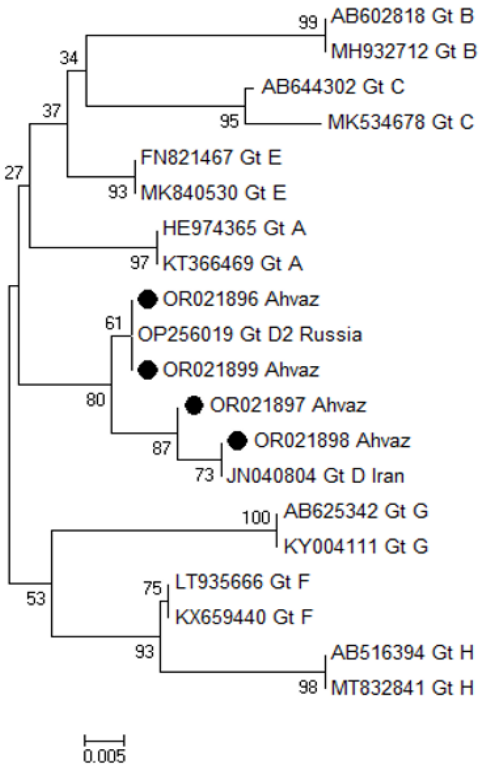

Figure 1. A Phylogenetic Tree Using the Neighbor Joining Method was Constructed to Compare Partial Sequences of the S Region of HBV Isolates from Patients in Ahvaz City with 8 Different HBV Genotypes. The corresponding accession numbers were retrieved from GenBank, and the genotypes were isolated from various regions around the world. The constructed phylogenetic tree demonstrated the clustering of all four HBV isolates (accession numbers OR021896-OR021899) from Ahvaz city, marked with black circles, with genotype D2 from Russia (OP256019) and genotype D from Iran (JN040804). The accuracy of the phylogenetic tree was evaluated by conducting 1000 bootstrap replicates. The scale bar was set at 0.005.

Table 1. Displays the Age Distribution among Breast Cancer Patients with Varying Cancer Types

Age grouping	Cancer types					P value
Range 29-80	Ductal	Lobular	Mucinous	Medullary	Metastatic	
29	1 (1.11%)	--	--	--	--	
30-39	13 (24.44%)	1 (1.11%)	2 (2.22%)	1 (1.11%)	--	
40-49	25 (27.77)	4 (4.44%)			1 (1.11%)	0.315
50-59	22 (24.44%)	5(5.55%)	--	1 (1.11%)	--	
60-69	11 (12.22%)	--	--	--	--	
70-79	3 (3.33%)	--	--	--	--	
> 80	--		--	--	--	

Table 1 presents the prevalence of breast cancer across diverse age cohorts, with the minimum incidence of 1.11% noted in persons below the age of 29, while the maximum incidence of 27.77% was observed in the 40-49 age bracket for ductal carcinoma. Moreover, the prevalence of lobular carcinoma varied from 1.11% in individuals aged above 80 years to 5.55% in those belonging to the 50-59 age group. Nevertheless, these results were not statistically noteworthy (p=0.315).

rate of 5.55% for hepatitis B virus (HBV) markers among the individuals diagnosed with breast cancer. Wu et al. [43] have documented the occurrence of HBV within a cohort consisting of 2796 females diagnosed with breast cancer, revealing a prevalence rate of 8.36% (with 234 individuals testing positive for HBsAg). These findings exhibited an elevated magnitude in comparison to our own outcomes.

In a separate investigation, Lu et al. [44] disclosed their discoveries concerning the frequency of favorable HBsAg in a total of 2452 instances involving patients diagnosed with breast cancer in the southwestern region of China, yielding a recorded proportion of 8.2%.

Wang et al. [45] conducted an investigation involving 236 cases of breast cancer patients afflicted with hepatitis B virus (HBV) infection, who underwent chemotherapy. Among these cases, a total of 44 (18.6%.) HBV reactivation were identified. In our investigation, a total of 1.11% of individuals exhibited ductal carcinoma and tested positively solely for anti-HBc. This finding suggests a potential presence of seropositive occult HBV infection, thus emphasizing the need for additional molecular detection methods.

While, Lu et al. [44] observed that the high occurrence of positive anti-HBc was 66.4% in breast cancer patients in China. Drawing from these results, the authors propose the need to account for occult hepatitis B infection in breast cancer patients who test positive for anti-HBc but negative for HBsAg. Baghbanian et al. [46] have documented that out of the 204 blood samples of patients with hematological cancers and solid cancers who were negative for HBsAg, 11 samples (5.4%) were found to be positive for anti-HBc. HBV-DNA was identified in 9 out of the 11 samples (81%) that tested positive for anti-HBc in Iran. The prevalence of occult HBV infection in hematological cancers was higher compared to solid cancers, with rates of 4.8% and 4.3% respectively.

The findings of this study indicate that the prevalence of HBV markers, specifically at a rate of 5.5%, is comparatively higher among women diagnosed with breast cancer when compared to the prevalence of HBsAg carriers, which stands at a rate of 2.2%, in the Iranian population [21].

Given that breast cancer patients may harbor HBV infection and subsequently undergo surgery and chemotherapy, the repercussions of HBV reactivation can manifest as acute fulminant hepatitis, liver failure, and mortality [25, 26]. Hence, it is imperative to introduce HBV serology screening and molecular examinations such as PCR or Real-time PCR for female patients suffering from breast cancer prior to their surgical and chemotherapeutic interventions. At the current time, there is currently no efficacious remedy accessible for combating the hepatitis B virus, whereas the sole preventative measure lies in the HBV immunization.

Since 1993, the nationwide vaccination campaign has brought about a transformation in the prevalence of HBV infection within Iran, reducing it from 3.6% to a

Table 2. Distribution of HBV Markers in Patients with Various Type of Breast Cancer

Categories	HBV markers			cancer types			
Age 29-80	HBsAg	anti-HBc	HBV PCR	ductal invasive	ductal in situ	lobular	histological grade
34	+	+	+	-	-	+	II
41	+	+	+	+	-	-	III
43	+	+	+	+	-	-	II
52	+	+	+	+	-	-	II
51	-	+	-	+	-	-	II

Table 2 exhibits among the cancer patients, a total of 5 out of 90 (5.55%) individuals were identified to have HBV markers (HBsAg, anti-HBc, HBV PCR). Out of these cases, 3 (3.33%) were diagnosed with ductal carcinoma (one histological grade III, and two histological grade II) while 1 (1.11%) had lobular carcinoma (histological grade II). Remarkably, 1 (1.11%) patient with ductal carcinoma (histological grade II) exclusively exhibited anti-HBc positivity.

low endemic level of 2.2% [47]. Despite the utilization of immunization, the occurrence of perinatal infection with HBV remains a plausible scenario. Furthermore, the existence of alterations in the "a" determinant of HBV can lead to the ineffectiveness of the vaccine [48].

In Taiwan, 10.9% of HBV-vaccinated children were found to have occult HBV infection [49]. Therefore, it is imperative to intensify HBV screening and monitoring of the effectiveness of HBV vaccination from the neonatal stage, as this is likely to prevent or delay the onset of breast cancer. The World Health Organization (WHO) has put forth a proposal to eradicate the hepatitis B virus on a global scale by the year 2030 [50]. Nevertheless, the successful execution of this program poses a significant challenge, particularly for individuals of the general public above the age of 30 with chronic HBV infection or occult hepatitis B infection (OBI) who may be susceptible to breast cancer in low and middle-income nations where the initial vaccination was initiated 29 years prior [51].

Several studies have indicated that prophylaxis with lamivudine significantly reduces the rate of hepatitis B virus (HBV) reactivation in patients with breast cancer, despite the potential consequences [24, 52]. Conclusion, the examination of the data did not yield any significant results (p= 0.315) in the distribution of cancer types among different age groups. Within the population of individuals diagnosed with breast cancer, a noteworthy prevalence of 5.5% was observed in relation to HBV markers. The prevailing HBV genotype among breast cancer patients was determined to be genotype D. To minimize the potential of HBV reactivation during the immunosuppressive stage of chemotherapy patients, it is strongly advised that screening for serological HBV markers, including HBsAg and anti-HBc by ELISA, as well as HBV DNA tests by Real-time PCR, be performed on the serum of patients with breast cancer.

Author Contribution Statement

Study concept and design, Manoochehr Makvandi, and Mehdi Parsa Nahad, Analysis and interpretation of data, Roya Pirmoradi, Abdolhasan Talaeizadeh, Drafting of the manuscript Venus Fakheri sueini, Roya Pirmoradi; Critical revision of the manuscript for important intellectual content, Elena Lak, Amirmohamad Alborzi Ali Mohammad Arabzadeh; Statistical analysis, Maryam Moradi.

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Ethical consent

The project was endorsed by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, with the ethical code IR.AJUMS.MEDICINE.REC.1401.058. All patients included in the study were verbally informed of the purpose of the study and invited to participate on a voluntary basis. Written informed consent was collected from all patients,

Conflict of Interests

All authors declared that they have no conflict of interest.

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