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Original Research Paper

Methylation Status of the *RB1* Gene as a Diagnostic and Prognostic Tool in Breast Cancer: Correlations with Clinicopathological Features and Immunohistochemical Markers

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Abstract: The *RB1* gene plays important role in regulating the cell cycle and has been implicated in various malignancies, including breast cancer. Methylation-specific PCR (MSP) was used to assess *RB1* methylation, while immunohistochemical markers such as ER, PR, HER2, and Ki-67 were analyzed to correlate molecular changes with clinical characteristics. The findings demonstrate that aberrant *RB1*methylation is significantly linked to more aggressive tumor phenotypes, including higher histologic grade, advanced stage, and elevated Ki-67 expression. Furthermore, *RB1* methylation status correlated with poor survival outcomes, suggesting its role as a prognostic biomarker. This study underscores the importance of *RB1* methylation in breast cancer pathophysiology and its potential clinical utility.
Method: A total of 40 primary breast carcinoma tissue samples and 10 non-neoplastic breast tissue samples were collected from the pathology archives of medical institutions in Duhok, Iraq. Clinical and pathological data, including patient age, tumor size, stage, and grade, were retrieved from medical records. Hematoxylin and Eosin (H&E) staining was performed to confirm diagnosis. Immunohistochemical (IHC) staining was conducted for Ki-67, ER, PR, and HER2, while MSP was employed to evaluate *RB1* methylation and its association with clinical parameters.
Results: All patients in this study were female, with 70% being over 50 years old. Immunohistochemical analysis revealed that ER, PR, and HER2 were negative in 65%, 70%, and 60% of cases, respectively, while 70% of tumors exhibited high Ki-67 expression. A significant correlation was found between Ki-67 expression and receptor status, as well as other clinicopathological features. *RB1* methylation was identified in 24 cases (60%), demonstrating a strong association with tumor aggressiveness and immunohistochemical markers.**Conclusion:** The study confirms the association between *RB1* methylation and breast cancer progression. Evaluating *RB1* methylation status could provide valuable diagnostic and prognostic insights, potentially guiding targeted therapeutic strategies.

Keywords: Breast Cancer, *RB1*, Ki-67, TNBC

1. Introduction

Breast cancer remains a leading cause of cancer-related morbidity and mortality worldwide. While numerous molecular subtypes have been identified, the disease's complexity continues to challenge diagnostic and prognostic assessments [1]. The *RB1* gene, located on chromosome 13q14.2, encodes the retinoblastoma protein (pRB), a key regulator of cell cycle progression and genomic stability. Loss of *RB1* function, due to genetic mutations or epigenetic modifications such as promoter methylation, has been widely reported in triple-negative breast cancer (TNBC), contributing to uncontrolled proliferation and genomic instability [2]. *RB1* promoter methylation is implicated in breast tumorigenesis, correlating with specific biological and

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clinicopathological traits and serving as a potential prognostic and therapeutic target. Hypermethylation of CpG islands within tumor suppressor gene promoters often leads to gene silencing, as observed in *RB1* -altered tumors [3]. *RB1* protein expression is frequently absent or significantly reduced in methylated tumors, reinforcing the concept of epigenetic silencing. Given its high specificity and sensitivity, *RB1* methylation analysis may be a useful biomarker across various clinical settings [4].

Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype. Despite the progress made in precision treatment of cancer patients, targeted treatment is still at its early stage in TNBC, and chemotherapy remains the standard treatment. Encompasses a subset of breast cancers that lack expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). TNBC accounts for 15% to 20% of newly diagnosed breast cancer cases, and is known for its aggressive biological behavior and poor patient outcomes compared with hormone receptor-positive breast cancer. It has also been of special interest to breast cancer researchers due to its varying response towards standard chemotherapy, and its lack of other effective treatment options [5].

Ki-67 is a nuclear protein associated with proliferation of tumor cells known as a prognostic factor for breast cancer [6]. The correlation between RB1 and Ki-67, a well-known marker of cell proliferation, offers valuable insights into breast cancer biology. Studies have shown that RB1 inactivation, often caused by genetic mutations or promoter methylation, is associated with increased Ki-67 expression, reflecting higher rates of cell proliferation [7]. This relationship arises because RB1 is a critical regulator of the G1-to-S phase transition in the cell cycle, and its loss results in unchecked tumor growth. The association is particularly pronounced in aggressive subtypes like triple-negative breast cancer (TNBC), where RB1 loss and high Ki-67 levels are common, indicating a highly proliferative and poor-prognosis phenotype [8].

2. Methodology

Sample Collection

The present cross sectional study use archival formalin fixed paraffin embedded breast tissue samples obtained from pathology departments of medical centers in city of Duhok, Iraq. This collection included 40 histopathologically confirmed invasive breast carcinoma and 10 matching non-neoplastic control tissues from tumor adjoining areas of the same patients.

Immunohistochemistry (IHC)

Applying the auto-immunohistochemical technique (Dako Denmark, Link48). Unstained representative tumor sections, with little or no necrosis, were cut and mounted on poly- l - lysine-coated slides. After dewaxing, endogenous peroxidase activity was blocked by incubating tissue sections with methanol containing 3% hydrogen peroxide for 20 min, then they were processed according to the manufacturer's instructions (Dako Denmark, A/S), using the autostainer (Dako Denmark, Link48). The markers used included Estrogen Receptors (ER: monoclonal antibodies, clone EP1) and Progesterone Receptors (PR: monoclonal antibodies, clone PgR, 636). The primary antibodies used were monoclonal anti-Her2 (4B5) antibodies. After washing, sections were incubated with a biotinylated secondary antibody and treated with streptavidin-conjugated horseradish peroxidase (HRP), and the immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) as the chromogen.

Methylation Analysis

DNA extraction and bisulfite modification

For molecular analysis, tissue sections were prepared into paraffin blocks, followed by DNA extraction through proteinase K digestion. The concentration and purity of the extracted genomic DNA were measured in duplicate using a NanoDrop spectrophotometer and gel electrophoresis. After storage at -20°C, the DNA samples were treated with sodium bisulfite using the EpiTect Bisulfite Kit from Qiagen, which facilitated the conversion and purification of the DNA for subsequent methylation analysis.

Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) methods used where

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the CpG sites residing within the primer sets were used as a proxy for the methylation status of the region of interest. In the used MSP, the target (RB1 tumor suppressor genes) DNA was amplified with the mutation-specific primers near the promoter region of RB1 (MF: 5′-GGGAGTTTCGCGGACGTGAC-3′ and MR: 5′- ACGTCGAAACACGCCCCG-3′, PCR product size: 172 bp). The unmethylated DNA sequence was amplified using a primer specific to the unmethylated-bisulfite-converted DNA sequence, in which the C's (cytosines) in the template should be treated as T's (UF: 5′- GGGAGTTTTGTGGATGTGAT-3′ and UR: ′-ACATCAAAACACACCCCA-3′′, PCR product size: 172 bp). The methylation and unmethylation-specific primers were adapted from [9]. MS-PCR reactions were performed in 20µl total volume which contained 0,5µl MF primer and 0,5µl MR primer (synthesized by Macrogen, South Korea), 8µl of Hot Reaction Master mix (Addbio, Korea), and 2µl template of bisulfite converted DNA and this was made up to a final volume with DNase-free water. The cycling conditions were initial denaturation at 96oC for 7 min followed by 35 cycles of denaturation at 95o C for 1 min, annealing at 55o C for 1 min, and extension at 72oC for 1 min, followed by a final extension at 72oC for 5 min, using a thermocycler (Applied Biosystems USA). The PCR products were separated in 1.5% agarose gel electrophoresis after staining with Prime Safe Dye. The molecular findings were directly visualized under UV illumination.

Statistical Analysis

Statistical analyses were performed using version 22 of IBM SPSS Statistics software. The association between RB1 methylation status and clinicopathological features, as well asimmunohistochemical markers, was assessed using (Chi-square test). A p-value of <0.05 was considered statistically significant.

3.Results and discussion

Clinicopathological features of breast cancer patients

All patients were women, their ages ranged from 18 to 83 years (median: 50.5 years). (Table.1) summarizes cancer cases parameters. All cases were grade 3 infiltrative duct carcinoma (IDC). Of these (70%) were above 50 years of age. For tumor (T) and nodal (N) considerations, variable rates were distributed but collectively 49% were within T3 status and 50% were reported among N2 status. A light microscopic view of grade 3 infiltrative duct carcinoma is shown in (Figure 1). Immunohistochemically, cases were registered as negative ER, PR and HER2 tumors in this study were 65%, 70% and 60% respectively. High Ki67 proliferative index was observed in 70% of tumor cells (Figure 2).

Table 1: Cancer cases parameters

Parameters		Cancer cases No (%)
Age	<50	12 (30)
	>50	28 (70)
TN	T1N1	1 (3)
	T1N2	1 (3)
	T1N3	1 (3)
	T2N1	3 (7)
	T2N2	12 (30)
	T2N3	2 (5)
	T3N1	5 (12)
	T3N2	7 (17)
	T3N3	8 (20)
ER	Positive	14 (35)
	Negative	26 (65)
PR	Positive	12 (30)
	Negative	28 (70)
Her2/neu	Positive	16 (40)
	Negative	24 (60)
Ki-67	High	29 (73)
	Low	11 (27)

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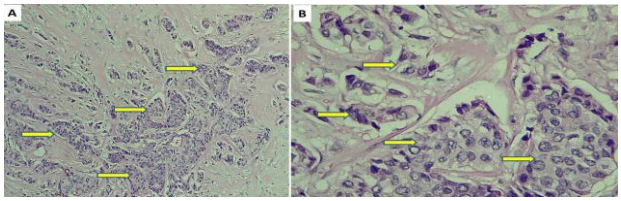

Fig1. Microscopic appearance of grade-3 infiltrative duct carcinoma (IDC). The malignant sheets are pointed by yellow arrows (H&E: A, X100; B, X400).

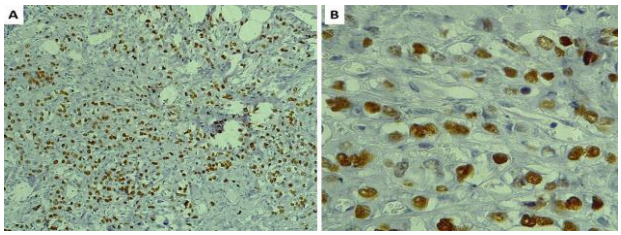

Fig 2. High proliferative index (Ki67) in infiltrative duct carcinoma (IHC: A, X100; B, X400)

RB1 Promoter Methylation

As shown in Table 2, *RB1* promoter methylation status in cancer cases revealed 24 (60%) cases of methylation (complete CM & partial PM) and 16 (40%) cases of un-methylation, while the controls showed 2 (20%) of complete methylation. Statistically, the difference of methylation between the malignant and control cases was significant between the control groups. Of the cancerous women with high expression of Ki-67 total methylation seen in 26 cases among 29 cases 65%. The difference was statistically significant (Table 3). The CM and PM methylation on gel electrophoresis results of some cases are shown in Figure 3.

Table 2: RB1 Methylation status among cancerous and control tissues

	RB1 Methylation			
	Methylated No (%)	Un-methylated No (%)	Total	P value
Malignant	24 (60)	16 (40)	40 (100%)	0.02
Control	2 (20)	8 (80)	10 (100)	

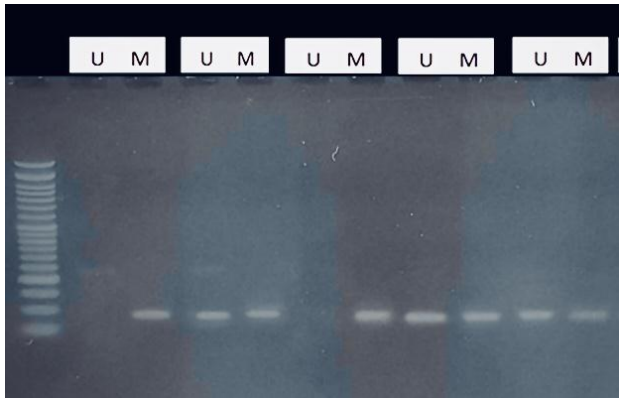

Fig 3. Methylation-specific PCR of the RB1 promoter region in tumor samples. Electrophoresis of Amplification Products Specific Promoter Region RB1 from Bisulfite-Treated DNA in Human Tumor Tissues. Each line reflects amplification products from methylated (M), 172bp or unmethylated (U), 172bp primer. The presence of a visible PCR product in lane (U) indicates the presence of unmethylated RB1 genes, and the presence of a product in lane (M) indicates the presence of methylated RB1 genes. Sample no. 1, 3, have complete methylated (CM) promoter region of the RB1 gene and sample no. 2, 4, 5 has partial methylated (PM) promoter region of the RB1 gene. The ladder used in the first line is 100.bp.

Table 3: *RB1* methylation and *Ki67* status among malignant cases

		RB1		Total	P value
		Methylated No (%)	Un-methylated No (%)		
Ki67	High	26 (65)	3 (8)	29 (73)	0.008
	Low	4 (10)	7 (17)	11 (27)	
	Total	40 (100)		40 (100)	

The positive *RB1* promoter methylated cases were significantly high among triple negative (ER-, PR-, Her2-) cases with a high proliferative index. Regarding T-status, methylation trended toward cases with T2 and more advanced tumors but didn't reach the level of significance. Also, there was a trend of methylation toward cases N2 cases but did not reach the level of significance (Table 4).

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Table 4: *RB1* methylation and the studied parameters.

		RB1			P value
		Methylated No (%)	Un-methylated No (%)	Total	
ER	Positive	5 (13)	9 (22)	14 (35)	0.04
	Negative	18 (45)	8 (20)	26 (65)	
PR	Positive	4 (10)	8 (20)	12 (30)	0.04
	Negative	19 (48)	9 (22)	28 (70)	
Her2/neu	Positive	4 (10)	12 30	16 (40)	0.004
	Negative	18 (45)	6 (15)	24 (60)	
TN	T1N1	0 (0.0)	1 (3)	1 (3)	0.5
	T1N2	0 (0.0)	1 (3)	1 (3)	
	T1N3	1 (3)	0 (0.0)	1 (3)	
	T2N1	3 (7)	0 (0.0)	3 (7)	
	T2N2	8 (20)	4 (10)	12 (30)	
	T2N3	2 (5)	0 (0)	2 (5)	
	T3N1	5 (12)	0 (0.0)	5 (12)	
	T3N2	7 (17)	0 (0.0)	7 (17)	
	T3N3	8 (20)	0 (0)	8 (20)	

After grouping of the tested markers, *RB1* methylation was high among ER-/PR-/Her2- (*38%*) and 30% 0f those were triple negative with high Ki-67 reaction. However, adding the proliferative index value, the difference did not reach the level of significance with *p*= (Table 5).

Table 5: Positive *RB1* methylation, Ki67 association with hormone receptors groups

Hormones Groups	**Methylated**	**Un-methylated**	**Total**
ER+/PR+/Her2/neu+	4 (10%)	3 (8%)	7 (18%)
ER+/PR+/Her2/neu+/High Ki67	2 (5%)	2 (5%)	4 (10)
ER+/PR+/Her2/neu+/low Ki67	2 (5%)	1 (3%)	3 (8%)
ER+/PR+/Her2/neu-	2 (5%)	5 (13%)	7 (18%)
ER+/PR+/Her2/neu-/High Ki67	1 (3%)	4 (10%)	5 (13%)
ER+/PR+/Her2/neu-/low Ki67	0 (0%)	2 (5%)	2 (5%)
ER-/PR-/Her2/neu+	3 (8%)	2 (5%)	5 (13%)
ER-/PR-/Her2/neu+/High Ki67	4 (10%)	1 (3%)	5 (13%)
ER-/PR-/Her2/neu+/low Ki67	0 (0%)	0 (0%)	0 (0%)
ER-/PR-/Her2/neu-	15 (38%)	6 (15%)	21 (53%)
ER-/PR-/Her2/neu-/High Ki67	12 (30%)	5 (13%)	17 (43%)
ER-/PR-/Her2/neu-/low Ki67	2 (5%)	2 (5%)	4 (10%)

Breast cancer prognosis depends on factors such as tumor size, grade, and receptor status [10]. The present study indicates that the majority of cancer cases occurred in older individuals (63.3%), a finding consistent with research conducted in Iran [11]. However, this contradicts the findings of [12], which suggested that those under 50 are neither at low risk nor protected.

Concerning the TN, T2N2 (30%) represented the greatest percentage, aligning with the findings of [13], however contradicting the results of [14], who reported that the majority of patients were identified as T3N1.

This study revealed the negative expression of the receptors ER, PR, and Her2 in cancer samples to 65%, 70% and 60% respectively, aligning with results from other study done locally by [15], but it's contraindicated with various studies [16-17].

RB1 is a known regulator of cell-cycle control; however, recent studies identified critical functions in regulating cancer associated gene networks that influence the DNA damage response, apoptosis, and cell metabolism [18].

In the current study *RB1*promoter methylation was significantly more prevalent in malignant breast tissues (60%) than in controls (20%). This aligns with [19], who linked RB1 loss to aggressive breast cancer subtypes, particularly TNBC. Additionally, [20] reported that *RB1*gene silencing via methylation contributes to tumor progression and therapy resistance.

This study showed strong association was observed between *RB1*methylation and high Ki-67 expression (p=0.008). [21] found that RB1-deficient tumors exhibit increased proliferation, leading to elevated Ki-67 expression.

Furthermore, *RB1*methylation was significantly associated with TNBC status (p=0.004), corroborating studies by [22] which linked RB1 loss to TNBC aggressiveness and poorer survival outcomes.

The findings from this study highlight a significant association between RB1methylation status, Ki67 expression, and hormone receptor subtypes in breast cancer. Notably, RB1 promoter methylation was predominantly observed in triple-negative breast cancer (TNBC) cases (ER-/PR-/HER2-), with 38% of methylated samples belonging to this subgroup. This aligns with researches indicating that RB1inactivation is more frequent in TNBC, contributing to uncontrolled proliferation, aggressive tumor behavior, and poor prognosis [23]. This suggests that epigenetic inactivation of RB1may contribute to the aggressive nature of TNBC, a subtype characterized by poor prognosis and limited targeted therapy options [24].

A robust correlation between RB1methylation and high Ki67 expression was observed, especially within the

triple-negative group, where 30% of cases exhibited both high Ki67 levels and RB1methylation. Ki-67 is a well-established marker of cell proliferation, and its increased expression in RB1-methylated tumors further supports the notion that RB1gene silencing contributes to tumor progression by promoting uncontrolled cell division [25].

Collectively, these results propose that RB1methylation could serve as a potential biomarker for aggressive breast cancer phenotypes, particularly in triple-negative and highly proliferative tumors. Given the increasing interest in epigenetic therapies, targeting RB1methylation could represent a novel therapeutic approach for TNBC patients, who currently have limited treatment options.

Conclusion

The findings confirm that *RB1*promoter methylation is a key molecular event in breast cancer, particularly in highly proliferative and triple-negative tumors. Evaluating *RB1*methylation status could aid in early detection and prognostic assessment, with potential implications for targeted therapy strategies.

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Ethics

This study was conducted under approval by the Research Ethics Committee at Scientific Research Division, Duhok Directorate General of Health, Ministry of Health issued approval 28082024-7-12.

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