ARID3B EXPRESSION IN PRIMARY BREAST CANCER SAMPLES

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AYŞE SELCEN OĞUZ ERDOĞAN

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submitted by **AYŞE SELCEN OĞUZ ERDOĞAN** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Orhan Adalı
Head of Department, **Biology** _____

Assoc. Prof. Dr. A. Elif Erson Bensan
Supervisor, **Biology Dept., METU** _____

Examining Committee Members:

Assoc. Prof. Dr. Mesut Muyan
Biology Dept., METU _____

Assoc. Prof. Dr. A. Elif Erson Bensan
Biology Dept., METU _____

Assoc. Prof. Dr. Tülin Yanık
Biology Dept., METU _____

Assist. Prof. Dr. Mehmet Somel
Biology Dept., METU _____

Assist. Prof. Dr. Bala Gür Dedeoğlu
Biotech. Inst., Ankara University _____

Date: **15.12.2014**

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Ayşe Selcen OĞUZ ERDOĞAN

Signature:

ABSTRACT

ARID3B EXPRESSION IN PRIMARY BREAST CANCER SAMPLES

Oğuz Erdoğan, Ayşe Selcen
M. S., Department of Biology
Supervisor: Assoc. Prof. Dr. A. Elif Erson Bensan

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ARID3B (AT-rich interaction domain 3) is a member of the family of ARID proteins, which constitutes evolutionarily conserved transcription factors implicated in normal development, differentiation, cell cycle regulation and chromatin remodeling. In addition, ARID3B has been linked to cellular immortalization, epithelial-mesenchymal transition (EMT) and tumorigenesis. Given the emerging roles of ARID3B in tumor development, we examined its expression in primary patient-derived breast cancer samples to further investigate and elucidate the role of ARID3B in cancer pathogenesis and progression. Therefore we used immunohistochemical (IHC) staining procedure using formalin-fixed paraffin-embedded (FFPE) blocks of 63 primary breast cancer samples. We compared ARID3B IHC staining according to different cellular localizations with major prognostic factors of breast cancer. Our results show that ARID3B nuclear staining is positively correlated with ER status of the tumor and negatively correlated with tumor grade, mitotic activity and HER2 expression. This study is significant in terms of revealing an important and novel relationship between

ARID3B expression and ER and HER2 expression in breast cancer. Further studies will reveal the functional significance of the correlation between ER, HER2 and ARID3B expression which may improve our current knowledge of this relatively novel but potentially interesting protein.
Keywords: ARID3B, Breast Cancer, Immunohistochemistry (IHC)

ÖZ

PRİMER MEME KANSERİ ÖRNEKLERİNDE ARID3B EKSPRESYONU

Oğuz Erdoğan, Ayşe Selcen
Yüksek Lisans, Biyoloji Bölümü
Tez Yöneticisi: Doç. Dr. A. Elif Erson Bensan

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ARID3B, iyi korunmuş DNA bağlanma bölgesi içeren ARID ailesine bağlı bir proteindir. ARID3B'nin birçok fizyolojik ve patolojik süreçte önemli roller oynadığı yapılan çalışmalarla gösterilmiştir. Embriyonel gelişimde özellikle etkili olduğu düşünülen ARID3B, literatürde ortaya konan farklı deneyler ile birçok organ sisteminin gelişimiyle ilişkilendirilmiştir. Nöroblastoma ARID3B ile ilişkisi gösterilmiş, önemli ve sık görülen bir çocukluk çağı tümörüdür. Bu tümör tipinde ARID3B ekspresyon seviyelerinin tümör derecesi ile korele olarak değişiklik gösterdiği gösterilmiştir. Yine nöroblastomalar üzerinde yapılan bir çalışmayla ARID3B'nin bu hücrelerde apopitozu engellediği gösterilmiştir. Over tümörlerinde yapılan bir diğer çalışmada ise ARID3B'nin, nöroblastomalar üzerinde yapılan çalışmadan farklı olarak, TNFα yoluyla apopitozu indüklediği ortaya konmuştur. Grubumuz tarafından yapılan bir çalışmada ise ARID3B meme tümörlü hücre hatlarında, hücre hareketleri ile ilişkilendirilmiştir. Biz de çalışmamızda ARID3B'nin kanser oluşumu ve ilerlemesindeki etkilerini aydınlatabilmek için tüm dünyada kadınlarda en sık görülen kanser olan meme tümörlerinde ARID3B

protein ekspresyonunu immünhistokimyasal olarak araştırdık. Formalinle fikse edilmiş ve parafine gömülü 63 primer meme tümörü örneğinde yaptığımız çalışmamızda ARID3B'nin farklı lokalizasyonlardaki ekspresyonunun meme tümörünün tanımlanmış ve belli başlı prognostik parametreleri ile korelasyonunu karşılaştırdık. Çalışmamız sonucunda ARID3B'nin meme tümörlerinde ER ekspresyonu ile pozitif korelasyon göstermekle birlikte tümör derecesi, mitotik aktivite ve HER2 ekspresyonu ile negatif korelasyon gösterdiğini ortaya koyduk. Çalışmamızda çıkan sonuçlar ARID3B'nin meme tümörlerinde ER ve HER2 ekspresyonu ile ilişkisini ilk defa göstermesi açısından oldukça önemlidir. Ancak bu ilişkinin net olarak aydınlatılabilmesi için birçok farklı çalışmaya da ihtiyaç vardır.

Anahtar Kelimeler: ARID3B, Meme Kanseri, İmmünohistokimya (İHK)

To the memory of my little brother

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INTRODUCTION

1.1. Invasive Breast Cancer

Invasive breast cancer is the most common tumor type among women all over the world (Lakhani et al., 2012). Furthermore, it is the leading cause of cancer related deaths in women after lung cancer (Kumar et al., 2009).

The incidence of breast cancer starts to increase with puberty, sharply rises up during the reproductive years and levels off slightly after menopause. Still, breast cancer can be seen in a wide range of ages (Rosai, 2011).

The etiology of breast cancer, like many other cancer types, cannot be reduced or limited to a single cause, because of its complex and complicated nature (Lakhani et al., 2012). Environmental factors, dietary habits, hormonal influences and genetic predisposition are some of the main etiologic factors that play role to development of breast cancer (Kumar et al., 2009).

All breast tumors, with the exception of approximately 5% are adenocarcinomas (Kumar et al., 2009). With respect to World Health Organization (WHO) Classification, invasive breast carcinoma of no special type (NST) is the most seen histologic type of invasive breast cancer. There are also more than 20 rare epithelial tumor variants clearly described in this classification (Lakhani et al., 2012) (Table 1).

Table 1. WHO classification of breast tumors (Lakhani et al., 2012)

EPITHELIAL TUMORS	EPITHELIAL-MYOEPITHELIAL TUMORS
Microinvasive carcinoma	Pleomorphic adenoma
Invasivebreast carcinoma	Adenomyoepithelioma
Invasive carcinoma of no special-type (NST)	Adenomyoepithelioma with carcinoma
Pleomorphic carcinoma	Adenoid cystic carcinoma
Carcinoma with osteoclast-like stromal giant cells	PRECURSOR LESIONS
Carcinoma with choriocarcinomatous features	INTRADUCTAL PROLIFERATIVE LESIONS
Carcinoma with melanotic features	PAPILLARY LESIONS
Invasive lobular carcinoma	BENIGN EPITHELIAL PROLIFERATIONS
Classic lobular carcinoma	MESENCHYMAL TUMORS
Solid lobular carcinoma	FIBROEPITHELIAL TUMORS
Alveolar lobular carcinoma	Fibroadenoma
Pleomorphic lobular carcinoma	Phyllodes tumor
Tubulolobular carcinoma	Benign
Mixed lobular carcinoma	Borderline
Tubular carcinoma	Malign
Cribriform carcinoma	Periductal stromal tumor, low grade
Mucinous carcinoma	Hamartoma
Carcinoma with medullary features	TUMORS OF THE NIPPLE
Medullary carcinoma	Nipple adenoma
Atypical medullary carcinoma	Syringomatous tumor
Invasive carcinoma NST with medullary features	Paget disease of the nipple
Carcinoma with apocrine differentiation	MALIGNANT LYMPHOMA
Carcinoma with signet-ring-cell differentiation	METASTATIC TUMOR
Invasive micropapillary carcinoma	TUMORS OF THE MALE BREAST
Metaplastic carcinoma (NST)	CLINICAL PATTERNS
Low-grade adenosquamous carcinoma	
Fibromatosis-like metaplastic carcinoma	
Squamous cell carcinoma	
Spindle cell carcinoma	
Metaplastic carcinoma with mesenchymal differentiation	
Chondroid differentiation	
Osseous differentiation	
Other types of mesenchymal differentiation	
Mixed metaplastic carcinoma	
Myoepithelial carcinoma	
Rare types	
Carcinoma with neuroendocrine features	
Neuroendocrine tumor, well-differentiated	
Neuroendocrine carcinoma, poorly differentiated (small cell carcinoma)	
Carcinoma with neuroendocrine differentiation	
Secretory carcinoma	
Invasive papillary carcinoma	
Acinic cell carcinoma	
Mucoepidermoid carcinoma	
Polymorphous carcinoma	
Oncocytic carcinoma	
Lipid-rich carcinoma	
Glycogen-rich clear cell carcinoma	
Sebaceous carcinoma	
Salivary gland/skin adnexal type tumors	

1.2. Prognostic Factors of Breast Tumor

Like many other cancer types, the outcome of breast cancer depends on numerous pathological and clinical parameters (Rosai, 2011). Patients' age, grade and stage of the tumor, hormonal status of tumor cells and the lymphovascular tumor invasion are the most important indicators of the patient survey (Kumar et al., 2009; Lakhani et al., 2012). These prognostic factors guide the clinicians while choosing the right medical therapy for individual patients. Among the prognostic factors lymph node status is the most important parameter (Lønning, 2007).

1.2.1. Grading

All invasive breast tumors must be graded according to modified Nottingham Histologic Score by scoring tubule/gland formation, mitotic activity and nuclear pleomorphism (Lakhani et al., 2012). It is the "gold standard" grading system for breast tumors (Rakha et al., 2010). Patients' survival rates and biological characteristics of their tumors show very close relation with this scoring system, therefore microscopic grade is one of the important prognostic factors for breast tumors (Kumar et al., 2009; Rakha et al., 2010). Each parameter is evaluated and numbered from 1 to 3 and the sums of all three parameters are used to find the grade of the tumor. With this system, invasive breast cancer is subdivided into three groups: well (grade I), moderate (grade II) and poorly (grade III) differentiated (Kumar et al., 2009; Lakhani et al., 2012).

1.2.2. Staging

Tumor node metastasis (TNM) system is the most widely accepted staging system for breast cancer. The last update of this staging system was published by International Union for International Cancer Control (UICC)/American Joint Committee on Cancer (AJCC) at 2012 (Compton et al., 2012).

In this system T represents primary tumor size and extension, N shows lymph node involvement by tumor cells and M shows presence or absence of distant metastasis

(Compton et al., 2012). According to all three parameters, the stage of the tumor is determined and classified roughly into five (0 to IV) stages. Patients' survival rates indicate significant relation with these five stages. Patients with stage I disease mostly have the best prognosis; on the other hand, patients with stage IV disease have relatively the worst prognosis (Compton et al., 2012; Lakhani et al., 2012; Rosai, 2011). Latest TNM classification and stage groups are shown in Table 2 and Table 3, respectively.

Table 2. Pathologic TNM classification of breast tumors (Lakhani et al., 2012)

Primary Tumor (T)	**TX**	Primary tumor cannot be assessed	**Regional Lymph Nodes (pN)**	**pN2** pN2a pN2b	 Metastasis in 4-9 axillary lymph nodes, including at least one that is larger than 2 mm Metastasis in clinically detected internal mammary lymph node(s), in the absence of axillary lymph-node metastasis
	T0	No evidence of primary tumor			
	Tis 	Carcinoma in-situ Tis (DCIS) Tis (LCIS) Tis (Paget)			
	T1 T1mi T1a T1b T1c	Greatest dimension of the tumor ≤ 2cm Microinvasion (greatest dimension≤ 0.1cm) 0.1cm < greatest dimension ≤ 0.5cm 0.5cm < greatest dimension ≤ 1cm 1cm < greatest dimension ≤ 2cm		**pN3** pN3a pN3b pN3c	Metastasis in 10 or more axillary lymph nodes (at least one larger than 2 mm) or metastasis in infraclavicular lymph nodes Metastasis in clinically detected internal ipsilateral mammary lymph node(s) in the presence of positive axillary lymph-node(s); or metastasis in more than 3 axillary lymph nodes with microscopic or macroscopic metastasis detected by sentinel lymph-node biopsy but not clinically detected Metastasis in ipsilateral supraclavicular lymph node(s)
	T2	2cm < Greatest dimension of the tumor ≤ 5cm			
	T3	Greatest dimension of the tumor> 5cm			
	T4 T4a T4b T4c T4d	Tumor of any size with direct extension to chest wall and/or to skin Extension to chest wall Ulceration, ipsilateral satellite skin nodules or skin oedema Both 4a and 4b Inflammatory carcinoma			
Regional Lymph Nodes (pN)	**pNX**	Regional lymph nodes cannot be assessed	**Distant Metastasis (M)**	**M0**	No distant metastasis
	pN0	No regional lymph-node metastasis			
	pN1 pN1mi pN1a pN1b pN1c	Micrometastasis; or metastasis in 1-3 axillary ipsilateral lymph nodes; and/or in internal mammary nodes with metastasis detected by sentinel lymph-node biopsy but not clinically detected Micrometastasis (0.2mm < greatest dimension ≤ 2mm and/or more than 200) Metastasis in 1-3 axillary lymph node(s) including at least 1 larger than 2 mm in greatest dimension Internal mammary lymph nodes with microscopic or macroscopic metastasis detected by sentinel lymph node biopsy but not clinically detected Metastasis in 1-3 axillary lymph nodes and internal mammary lymph nodes with microscopic or macroscopic metastasis detected by sentinel lymph node biopsy but not clinically detected		**M1**	Distant metastasis

Table 3. Stage grouping of breast tumors (Lakhani et al., 2012)

	T	N	M
Stage 0	Tis	N0	M0
Stage 1A	T1	N0	M0
Stage 1B	T0, T1	N1mi	M0
Stage 2A	T0, T1 T2	N1 N0	M0
Stage 2B	T2 T3	N1 N0	M0
Stage 3A	T0, T1, T2 T3	N2 N1, N2	M0
Stage 3B	T4	N0, N1, N2	M0
Stage 3C	Any T	N3	M0
Stage 4	Any T	Any N	M1

1.2.3. Hormonal Status

Estrogen (ER) and progesterone (PR) receptor expression levels play crucial role in the tumor management (Lakhani et al., 2012). Invasive breast tumors, containing high levels of estrogen (ER) and progesterone receptors (PR) have better response to endocrine therapy (Rosai, 2011). Essentially, ER is the major biomolecule for prediction of tumor cells response to hormonal therapy over PR status. There is a strong correlation between percentages of ER positivity in tumor mass and disease free survival of patients (Viale et al., 2007). Still, PR expression in a tumor mass

(even very low levels) shows correlation with better clinical course (Lakhani et al., 2012; Mohsin et al., 2004). Consequently, the assessment of ER and also PR should include intensity of positive cells and proportion of these ER and PR positive cells to the whole tumor area (Lakhani et al., 2012).

Many different methods have been used to determine the levels of ER and PR in the tumor tissue. Nowadays, immunohistochemistry (IHC) is the generally accepted method for the evaluation of ER and PR status (Harvey et al., 1999; Lakhani et al., 2012).

ERBB2 (HER2) is a transmembrane protein which has tyrosine kinase activity and is the member of epidermal growth factor receptors family (Lakhani et al., 2012; Rosai, 2011). Although strong expression of HER2 is associated with poor outcome of disease, the primary objective of the routine evaluation of HER2 expression in a breast tumor is to determine appropriate candidates for HER2 targeted therapy (Kumar et al., 2009; Lakhani et al., 2012). Immunohistochemistry and/or fluorescence in situ hybridization (FISH) are two common methods for HER2 assessment (Rosai, 2011).

1.2.4. Lymphovascular Invasion

Lymphovascular invasion (vascular invasion or LVI) is described as the presence of tumor cells within the lumen of unequivocally defined blood or lymphatic vessels. Periphery of main tumor mass is mostly the best area for evaluation of LVI (Lakhani et al., 2012). Despite being an association between LVI and lymph node metastasis, LVI is a very important prognostic factor by itself, either presence or absence of lymph node metastasis (Lakhani et al., 2012; Mohammed et al., 2011; Song et al., 2011).

In addition to these well-established prognostic factors, researchers are investigating better and more powerful markers that may benefit the chemotherapy decisions and/or the well being of the patients. These studies not only help biomarker development but also provide valuable information about disease biology in terms of

deregulated genes and pathways. The focus of this thesis was also investigating a novel gene, ARID3B in breast cancers.

1.3. ARID3B

The ARID (AT-rich interaction domain) family is a group of proteins with evolutionary conserved DNA-binding domains composed of nearly 80 amino acid residues (Kortschak et al., 2000; Wilsker et al., 2002, 2005). In the literature, more than a dozen ARID family member proteins have been reported, with very important roles in cellular development, growth and differentiation processes, since their initial discovery in 1995 (Herrscher et al., 1995; Wilsker et al., 2005).

ARID family proteins are mainly subdivided into seven groups, according to their sequence identities: ARID1, ARID2, ARID3, ARID4, ARID5, JARID1 and JARID2 (Wilsker et al., 2004, 2005). Only two subgroups of ARID family proteins, ARID3 and ARID5, bind DNA with AT-rich consensus site. Other groups have no clearly defined sequence preference for DNA interaction (Wilsker et al., 2005).

ARID3 subgroup has three members: ARID3A, ARID3B and ARID3C. Uniquely ARID3 subgroup has an "extended" ARID (eARID) sequence with nearly 35 residues, immediately after the sequence of core ARID (Wilsker et al., 2004). In addition to that, eARID proteins also have another conserved region known as REKLES that is not present in any non-ARID protein in the C-term of the protein (Kim et al, 2007; Kortschak et al., 2000). REKLES domain comprises two distinct subdomains: REKLESα and REKLESβ (Tidwell et al., 2011). These two REKLES domains are responsible for nuclear import and export of ARID3A (Kim & Tucker, 2006). Further studies showed that REKLESβ domain is also needed for ARID3B and ARID3A interaction (Kim et al., 2007).

ARID3B was first described as a DNA-binding protein that can bind to retinoblastoma protein. ARID3B has high expression levels in placenta, testis and leukocytes compared to other tissues and cells in the body (Numata et al., 1999). ARID3B is located on chromosome 15*q*24 and it has a pseudogene that is located on

chromosome 1*p*31 (Numata et al., 1999). A microdeletion syndrome on chromosome 15*q*24 with patients who have developmental and growth abnormalities, genital and skeletal malformations, specific face features and many different clinical conditions such as congenital heart disease and intestinal atresia had been described in 2007. ARID3B is one of the candidate proteins that its lack of expression may cause some of the symptoms of this microdeletion syndrome because of its role in embryonic development (Magoulas & El-Hattab, 2012).

ARID3B is a very essential protein for early embryogenesis. There are studies showing that ARID3B null mutant mice have fatal cranial mesenchymal tissue and cardiovascular system defects (Takebe et al., 2006). In addition to that, ARID3B has been found to play a role in cytoskeletal structure and cell motility for limb maturation during embryonic development (Casanova et al., 2011).

ARID3B also has been linked to differentiation of stratified epithelial cells such as skin, cervix and esophagus. Besides ARID3B has been found to be related with neuronal development (Samyesudhas et al., 2014).

Recently, ARID3B expression levels in tumor tissues and its association with cancer signaling pathways has been gaining attention (Lin et al., 2014). ARID3B was proposed as one of the new targets for p53 which is a very well-defined tumor suppressor protein (Garritano et al., 2013). p53 is commonly mutated in all human cancer types (Kumar et al., 2009).

Neuroblastoma, a neural crest originated childhood tumor, has close relationship with ARID3B. ARID3B expression levels show alterations in a stage dependent manner in this disease. Consequently, ARID3B was suggested to be an important protein for malignant transformation of neuroblastoma (Kobayashi et al., 2006). Besides, ARID3B and MYCN protein coexistence was found to be highly oncogenic for malignant transformation of neural stem cells by suppressing apoptosis (Kobayashi et al., 2012).

In a study on serous ovarian cancer, high ARID3B expression was linked to miR-125a. Moreover, miR-125a and ARID3B interaction was proposed to play a role in epithelial and mesenchymal transition (Cowden et al., 2009). Furthermore, ARID3B was also shown to be targeted by miR-125b and was linked to cell motility in breast cancer cells in a study from our group (Akhavantabasi et al., 2012). miR-125a and miR-125b share the same seed sequence and therefore can target the same mRNA targets.

In another study on ovarian cancer, ARID3B was found to be related with apoptosis by inducing TNFα mediating signaling pathway (Joseph et al., 2012), contrary to findings of Kobayashi et al (2012).

1.4. Aim of the Study

ARID3B is a relatively new protein and accumulating evidence about ARID3B in the literature reveal its importance in both normal tissue development and also cancer pathogenesis and progression.

In this study, we used immunohistochemical (IHC) staining procedure using formalin-fixed paraffin-embedded (FFPE) blocks of 63 primary breast cancer samples to further investigate ARID3B's potential role in breast cancer and elucidate its potential relationship with other prognostic factors for breast tumors.

CHAPTER 2

MATERIALS AND METHODS

2.1. Identification of Patient Samples

All tumor samples were obtained from 63 female patients who had been diagnosed with invasive breast carcinoma of no special type (NST) and treated with modified radical mastectomy and axillar lymph node dissection during the period of 2007-2012 at the Diskapi Yildirim Beyazit Training and Research Hospital, Ankara, Turkey.

Pathological data including histologic tumor type, tumor grade, tumor size, number of metastatic regional lymph nodes and lymphovascular invasion and hormonal status of the tumor, were recorded according to WHO Classification of Tumors of the Breast, 2012 guidelines from archival slides (H&E-stained, ER, PR and HER2) of the patients.

Data of distant metastasis (M) could not be reached for most of the patients. For this reason TNM stage of patients could not be determined accurately and excluded from the study. Tumor size (T) and regional lymph node status (N) are evaluated individually.

The study was conducted in concordance with the local ethical guidelines and was reviewed by the Diskapi Yildirim Beyazit Training and Research Hospital ethics committee.

2.2. Identification and Evaluation of Histomorphological Parameters

2.2.1. Tumor Grade

Tumor grade was determined with respect to modified Nottingham Histologic score by evaluating tubule/gland formation, nuclear pleomorphism and mitotic activity. Tubule/gland formation is evaluated only counting tubules or ductal acini, which have clear central lumina with surrounding neoplastic cells, in all tumor slides (Lakhani et al., 2012).

Mitotic count was assessed according to number of the exact and clear mitotic figures in the most proliferative areas of the tumor slides by counting at least 10 high power field (HPF/40X objective) on the microscope (Lakhani et al., 2012). In the study we used an Olympus CX31 microscope (40X field diameter 0.50 mm).

Nuclear pleomorphism was evaluated based on nuclear shape and nuclear size differentiation between tumor cells and normal epithelial cells of the breast tissue (Lakhani et al., 2012). The most pleomorphic area of the tumor was chosen for assessing of the nuclear pleomorphism (Lakhani et al., 2012).

The cut-off numbers of parameters are summarized in Table 4:

Table 4. Grading of breast tumors (Lakhani et al., 2012)

Score	**Parameters**		
	Tubule Formation	**Nuclear Pleomorphism**	**Mitotic Count (Olympus CX31, HPF)**
1	> 75% of the tumor	Minimal variation	≤ 7 /10 HPF
2	10-75% of the tumor	Moderate variation	8-14 / 10 HPF
3	< 10% of the tumor	Marked variation	≥ 15 / 10 HPF
GRADE			
I (score: 3, 4, 5)		II (score: 6, 7)	III (score: 8, 9)

2.2.2. Tumor Size

Tumor size was classified into 4 groups according to WHO Classification of Tumors of the Breast, 2012 guidelines like summarized in Table 5.

The greatest dimension of the tumor was assessed during the gross examination of the tumor tissue and it was also checked in microscopic evaluation of the tumor.

Tumor tissues with multiple foci were excluded from the study in order to avoid any doubt that may occur about exact tumor size.

Table 5. Grading of breast tumors (Lakhani et al., 2012)

GRADE	DEFINITION
T1	The greatest dimension of the tumor < 2 cm
T2	2 cm < The greatest dimension of the tumor≤ 5cm
T3	The greatest dimension of the tumor > 5 cm
T4	Tumor of any size with direct extension to chest wall and/or to skin

2.2.3. Regional Lymph Nodes

Regional lymph node metastasis was subdivided into 4 groups, shown in Table 6, according to WHO Classification of Tumors of the Breast, 2012 guideline.

Table 6. Grading of breast tumors (Lakhani et al., 2012)

GRADE	DEFINITION
N0	No regional lymph-node metastasis
N1	Metastasis in 1-3 axillary lymph-node
N2	Metastasis in 4-9 axillary lymph-node
N3	Metastasis in 10 or more axillary lymph-node

2.2.4. Lymphovascular Invasion

All H&E stained tumor slides were assessed for presence or absence of lymphovascular invasion and patients were divided into 2 groups: negative or positive for lymphovascular invasion. For the evaluation of lymphovascular invasion

tumor periphery area was used. Spaces filled with tumor cells were carefully examined in terms of endothelial cells.

2.2.5. Hormonal Status

2.2.5.1. ER and PR

Evaluation of ER and PR status had been done according to American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2010, guideline.

Nuclear staining of 1% or more of the tumor cells was considered as ER and PR positivity threshold. ER and PR expression was examined in terms of staining intensity and prevalence of positive tumor cells. The cut-off numbers are summarized in Table 7 and Table 8. In addition to that, ER and PR IHC scores were also calculated using the sum of prevalence of positively stained tumor areas and intensity of positively stained tumor cells. According to that, ER and PR IHC scores were classified into four groups: no staining (score 0), weak (score 1-2), moderate (score 3-4) and strong staining (score 5-6).

Table 7. Evaluation of ER and PR staining intensity

SCORE	INTENSITY
Score 0	No staining
Score 1	Weak staining
Score 2	Moderate staining
Score 3	Strong staining

Table 8. Evaluation of ER and PR staining prevalence

SCORE	PREVELANCE
Score 0	0-1% of staining
Score 1	2-35% of staining
Score 2	36-70% of staining
Score 3	71-100% of staining

2.2.5.2. HER2

HER2 assessment was done according to ASCO/CAP Guidelines, 2007 and subdivided into four groups as shown in Table 9.

Table 9. Evaluation of HER2 staining

SCORE	HER2 STAINING PATTERN
Score 0	No staining
Score 1+	Weak incomplete membrane staining in any proportion of tumor cells or weak, complete membrane staining in less than 10% of invasive tumor cells
Score 2+	A weak to moderate membrane staining is observed in more than 10% and less than 30% of invasive tumor cells
Score 3+	Uniform and intense membrane staining of more than 30% of invasive tumor cells

2.3. Immunohistochemistry for ARID3B

A standard avidin-biotin-peroxidase method was used for immunohistochemical staining procedure. First, 3-5 µm sections were taken from formalin-fixed paraffin-embedded (FFPE) blocks best representing the tumor and put onto the slides covered with poly-l-lysine. Sections taken due to the physical deparaffinization process were incubated overnight at 36°C and incubated for 1 hour at 60°C the next day. After that, slides were incubated in xylene for 10 minutes three times for chemical deparaffinization. Then, in order to hydrate the tissues again, slides were kept in 96% ethanol for 10 minutes three times. After this process, slides were placed in distilled water for 5 minutes and were treated with citrate buffer (pH 6) in household microwave oven (530 watt) for 20 minutes in order to unmask the antigens. Next, slides were cooled down in room temperature for 30 minutes and washed with phosphate buffered saline (PBS) for 10 minutes. Afterwards, tissues treated with 3% hydrogen peroxide solution for 10 minutes and were washed twice with PBS for 5 minutes. Then, slides were treated with Ultra-V-Block protein block solution (Thermo Fisher Scientific, Rockford, IL) for 5 minutes and incubated with rabbit polyclonal anti-ARID3B antibody (1:50, ab76499, Abcam, Cambridge, MA) (Table

10) for 2 hours at room temperature. After that, slides were washed twice with PBS for 5 minutes and incubated with anti-polyvalent HRP (Ready-To-Use) secondary antibody solution (Thermo Fisher Scientific, Rockford, IL) for 10 minutes. Tissues washed twice with PBS for 5 minutes were treated with streptavidin peroxidase for 10 minutes. After washing with PBS, slides were incubated with DAP chromogen (DAB Plus Substrate System-Thermo Fisher Scientific, Rockford, IL) for 10 minutes. After the slides had been washed with distilled water for 10 minutes, they were counterstained with Mayer's haematoxylin for 30 seconds and slides were covered with a waterborne coating material.

Table 10. Dilution and manufacturer of the antibody

	Dilution	Positive Control	Manufacturer	Antibody Type
ARID3B (ab76499)	1/50	Testicle	Abcam	Rabbit polyclonal

2.4. Evaluation of Immunohistochemistry for ARID3B

Evaluation of immunohistochemistry for ARID3B was mainly based on intensity and prevalence of staining similarly ER and PR IHC staining evaluation. Localization of ARID3B staining in terms of cytoplasmic and nuclear was also recorded separately. Cytoplasmic and nuclear IHC scores were identified.

Testis tissue was used as a positive control to determine ARID3B expression according to antibody manufacturer's instructions. Primary antibody for ARID3B was excluded for the negative control samples to verify specific staining in patient samples. Both cytoplasmic and nuclear IHC score was calculated with the sum of prevalence of tumor area stained positively and intensity of tumor cells stained positively.

Table 11. Evaluation of ARID3B intensity

SCORE	**INTENSITY**
Score 0	No staining
Score 1	Weak staining
Score 2	Moderate staining
Score 3	Strong staining

Table 12. Evaluation of ARID3B prevalence

SCORE	**PREVELANCE**
Score 0	0-1% of staining
Score 1	2-35% of staining
Score 2	36-70% of staining
Score 3	71-100% of staining

Table 13. Evaluation of ARID3B nuclear and cytoplasmic IHC scores

	ARID3B IHC SCORE (sum of prevalence and intensity of positive cells)
No staining	0
Weak	1-2
Moderate	3-4
Strong	5-6

2.5. Statistical Analyses

For statistical analysis, the SPSS software, version 15.0 (SPSS, Chicago, IL, USA) was used. Numeric variables were summarized as mean ± standard deviation and median (min-max). Categorical variables were summarized as number and percentage. Mann-Whitney or Kruskal-Wallis tests were used to assess numeric variable difference between groups where appropriate. Chi-square test and Fisher's exact test were used to evaluate categorical variable differences between groups.

Relationship between numerical variables was analyzed with Spearman correlation coefficient. Level of significance was taken as "$p<0.05$".

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Evaluation of Prognostic Parameters of the Patient Samples

All the patients included in the study were females. The youngest patient was 29 years old and the oldest patient was 86 years old. The mean age of patients was calculated as 57 (±14.1).

Grade 2 tumors were the most common group in all cases in the study with 47.6% followed by grade 3 and grade 1 tumors, respectively.

According to tumor size (T) more than half of the cases were determined T2. T4 tumors were recorded as the least common group with 4.8% of cases.

Regional lymph node metastasis was detected in 44.4% of cases and not detected in 55.6% of cases.

ER, PR and HER2 expression were observed in 65.1%, 48.4% and 54% of the cases, respectively.

Lymphovascular invasion was diagnosed in nearly half of the cases.

Prognostic features of the cases, mentioned above very briefly, are shown in Table 14, Table 15, Table 16 and Table 17 with more details.

Table 14. Prognostic features of cases-1

Parameters	Score	Number of cases	(%)
Grade	1	8	12.7%
	2	30	47.6%
	3	25	39.7%
Mitotic Activity	1	22	34.9%
	2	24	38.1%
	3	17	27%

Table 15. Prognostic features of cases-2

Parameters	Score	Number of cases	(%)
T	T1	12	19%
	T2	35	55.6%
	T3	13	20.6%
	T4	3	4.8%
N	N0	28	44.4%
	N1	15	23.8%
	N2	13	20.6%
	N3	7	11.1%

Table 16. Prognostic features of cases-3

Parameters	**Score**	**Number of cases**	**(%)**
ER intensity	0	22	34.9%
	1	4	6.3%
	2	16	25.4%
	3	21	33.3%
ER prevalence	0	22	34.9%
	1	4	6.3%
	2	14	22.2%
	3	23	36.5%
PR intensity	0	32	51.6%
	1	2	3.2%
	2	20	32.3%
	3	8	12.9%
PR prevalence	0	32	50.8%
	1	9	14.3%
	2	14	22.2%
	3	8	12.7%
HER2	0	29	46.0%
	1	6	9.5%
	2	10	15.9%
	3	18	28.6%

Table 17. Prognostic features of cases-3

Lymphovascular Invasion	Number of Cases (%)
Present	31 (%49.2)
Absent	32 (%51.8)

3.2. Immunohistochemistry Results for ARID3B

Initially, ARID3B IHC was optimized on testis tissue using 1:50 dilution of ARID3B antibody. Next, the specificity of ARID3B IHC was evaluated by performing IHC without the primary antibody. Both figures of positive and negative controls are shown in Figure 1 and Figure 2, respectively.

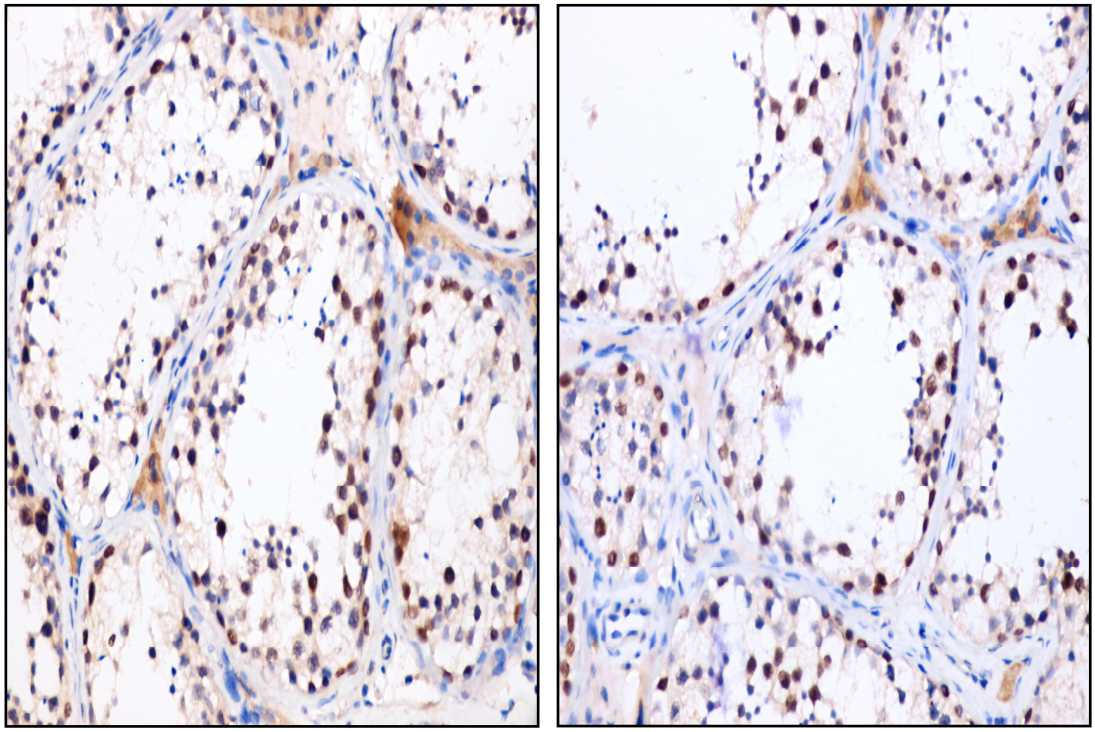
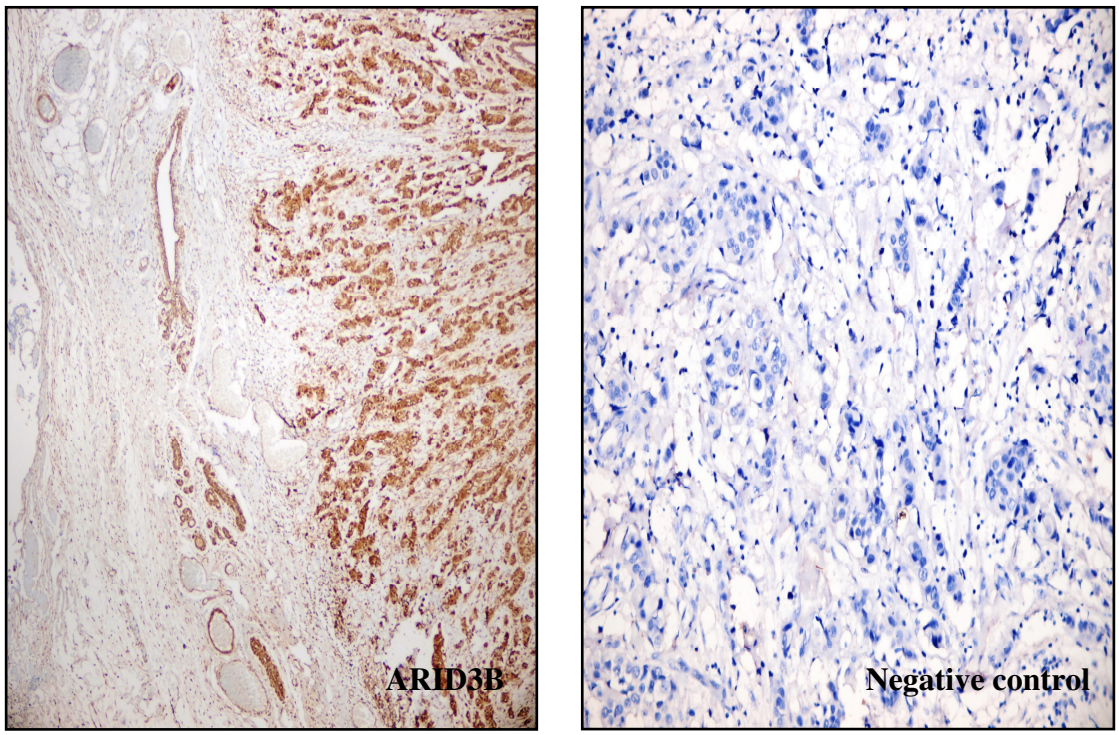
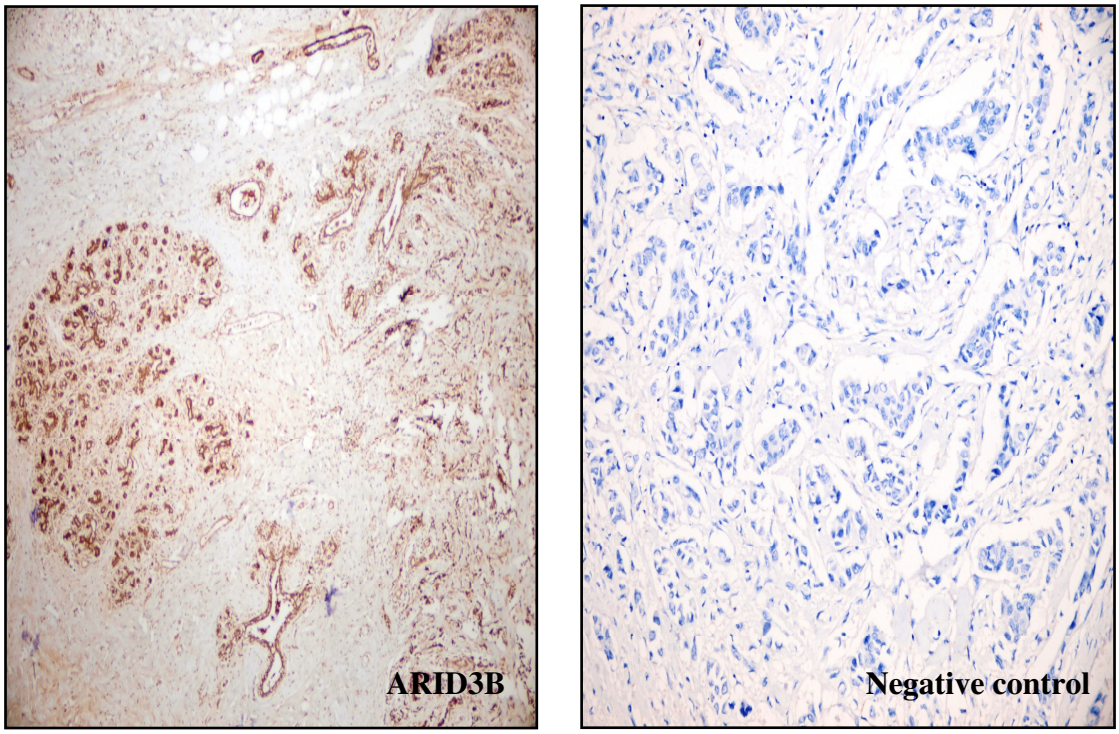


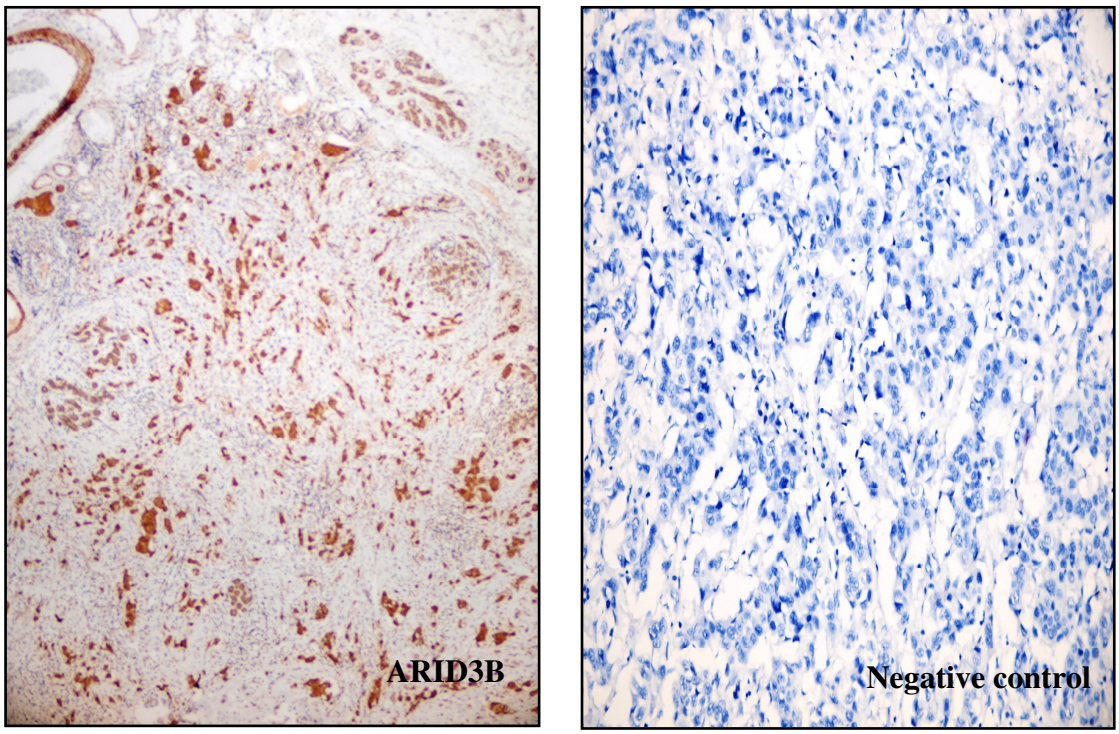
Figure 1. Positive staining of ARID3B in testis tissue as positive control (40X).



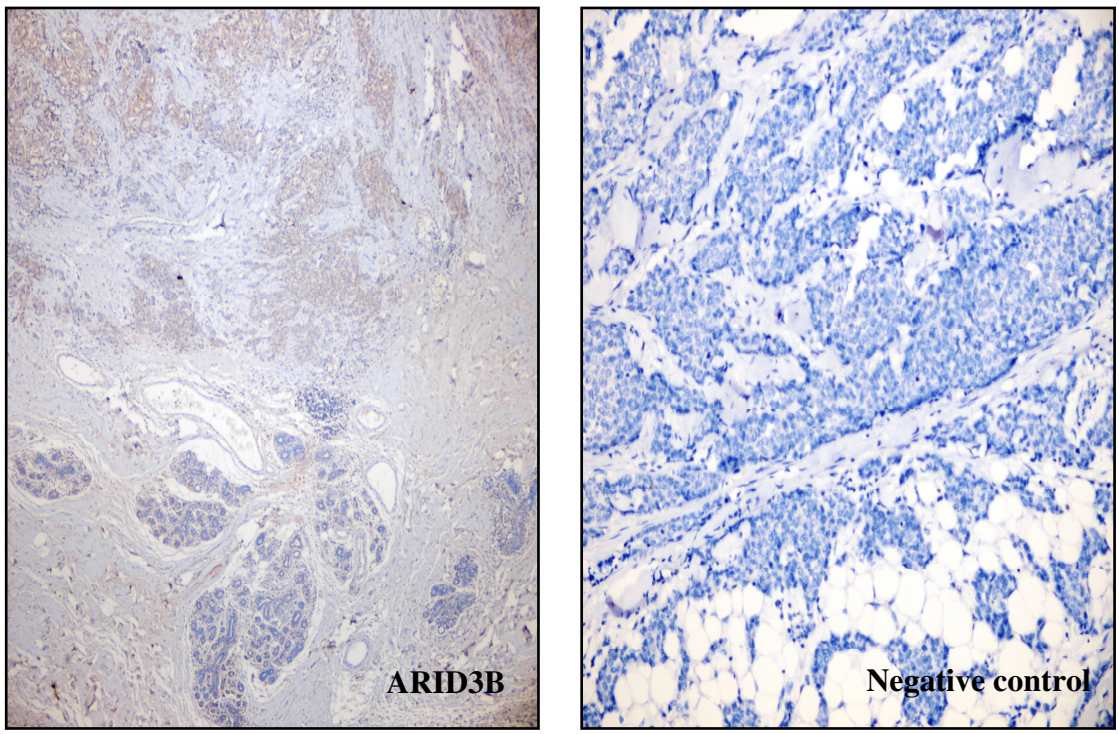
(a)



(b)



(c)



(d)

Figure 2. Four different tumor samples and their negative control samples; ARID3B primary antibody was excluded from the experiment to obtain negative controls (ARID3B 10X, Negative control 20X).

Different sub-cellular localization of ARID3B staining, including cytoplasmic, nuclear and membranous staining, was seen in all 63 invasive breast cancer samples. In all cases of the study, cytoplasmic staining of ARID3B was detected (63/63). Nuclear staining was noted only in 30% of the samples (19/63) and low level membranous staining was observed in 35% (22/63) of cases. ARID3B was initially defined as nuclear protein (Numata et al., 1999). However other studies and our results also suggest different sub-cellular localization of ARID3B.

To search for possible explanation, first we examined if there were different ARID3B isoforms that may result with alternate localization patterns. University of California Santa Cruz Genome Browser (UCSC) and National Center for Biotechnology Information (NCBI) databases were used for possible isoforms of ARID3B. According to the databases, there are 4 additional isoforms of ARID3B, these isoforms are of different molecular weights; 48 kDA, 28 kDA, 23 kDA and 14 kDA and they have 435, 253, 216 and 138 aminoacids, respectively. Full length ARID3B protein has 560 amino acids and is 61 kDA. The antibody that we used in this study binds the ARID3B full length protein between the 418^{th} and 447^{th} amino acids from its REKLES domain. Among the 4 isoforms, only 216 amino acid length and 23 kDA weight isoform can be recognized by the same antibody used in the study. In the literature, the 28 kDA isoform has been studied to have different sub-cellular localization patterns (Joseph et al., 2012). In the same study, Joseph et al also suggest ARID3B full length to be associated with the plasma membrane and cytoplasm (Joseph et al., 2012). In addition to that, ARID3B was also reported to be expressed in cytosol and cell membrane of both normal and malignant tissues by other researches (Joseph et al., 2012; Samyesudhas et al., 2014). Our results were in agreement with these prior studies.

Figure 3 shows the full length ARID3B and the 23 kDA isoform recognized by the antibody we used in the study.

Following the IHC staining of patient samples, we started analyzing IHC patterns with existing prognostic factors. There were no positive or negative correlations

between cytoplasmic and membranous ARID3B staining and any clinicopathological parameters for this group of patients.

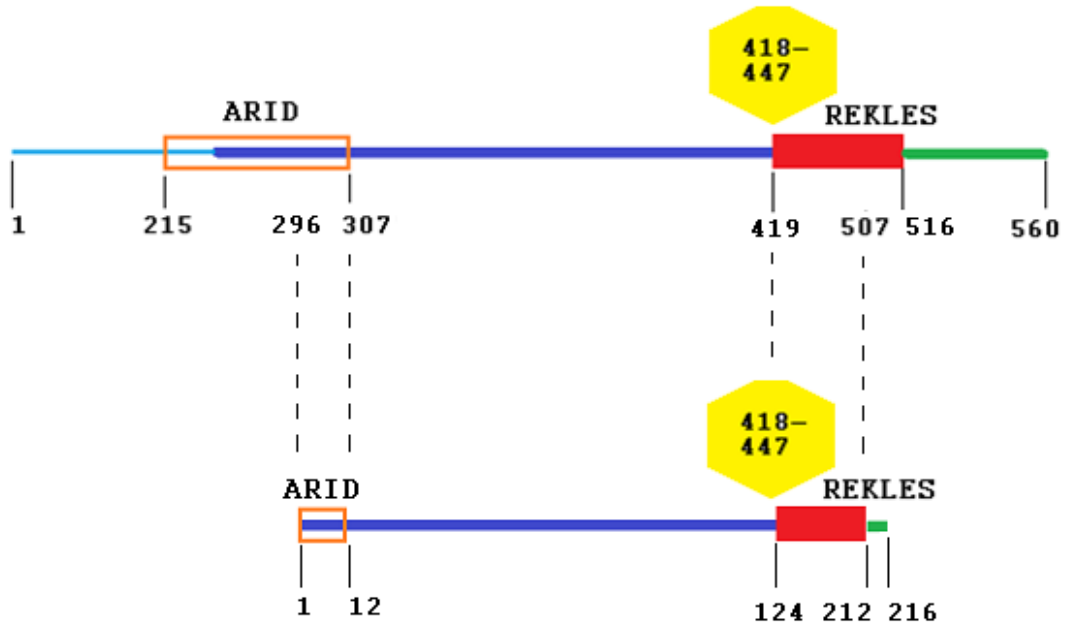


Figure 3. ARID3B full length (61 kDA) and the 23 kDA isoform

3.3. Comparison of Nuclear ARID3B Expression with Prognostic Parameters of Breast Tumor

Nuclear expression of ARID3B was significantly (positively or negatively) related with parameters including tumor grade, mitotic activity, and ER and HER2 expression. Nuclear expression of ARID3B indicated positive correlation only with ER expression, whereas other parameters such as tumor grade, mitotic activity and HER2 expression were negatively correlated with nuclear expression of ARID3B.

There were no correlations observed between patient age, tumor size, regional lymph node metastasis, lymphovascular invasion and PR expression with cytoplasmic, nuclear or membranous ARID3B expression. The results are summarized in Table 18.

Table 18. Comparison of nuclear ARID3B expression with prognostic parameters

Clinicopathological parameters		**ARID3B-Nuclear IHC Score**	
		Correlation coefficient	p-value
Age		- 0.038	0.765
Tumor grade		- 0.323	0.010*
Tubule formation		- 0.076	0.554
Mitotic count		- 0.368	0.003*
Tumor size (T)		- 0.178	0.163
Regional lymph node metastasis (N)		- 0.012	0.928
ER staining intensity		0.307	0.014*
ER staining prevalence		0.366	0.003*
PR staining intensity		0.243	0.057
PR staining prevalence		0.244	0.054
ERBB2 expression		- 0.275	0.029*
		Median (min - max)	p-value
Nuclear pleomorphism	Score 2	0 (0 - 4)	0.223
	Score 3	0 (0 - 3)	
Lymphovascular invasion	Positive	0 (0 - 3)	0.714
	Negative	0 (0 - 4)	

* Indicates significance

3.4. Relation of ARID3B Expression with ER Expression of the Breast Tumor Samples

ARID3B nuclear expression indicated positive correlation both with ER expression intensity and prevalence as shown in Table 18. Particularly, ER positivity was

observed in 89% (17/19) of nuclear ARID3B positive cases. Nuclear ARID3B and ER expression can be examined in representative cases of Figure 4 to Figure 8. Figure 4 and Figure 5 represent tumors with high ER staining and prevalence and high nuclear ARID3B. Figure 6 and Figure 7 represent tumors with moderate ER and moderate ARID3B and Figure 8 represents a tumor with no ER staining and very weak ARID3B expression.

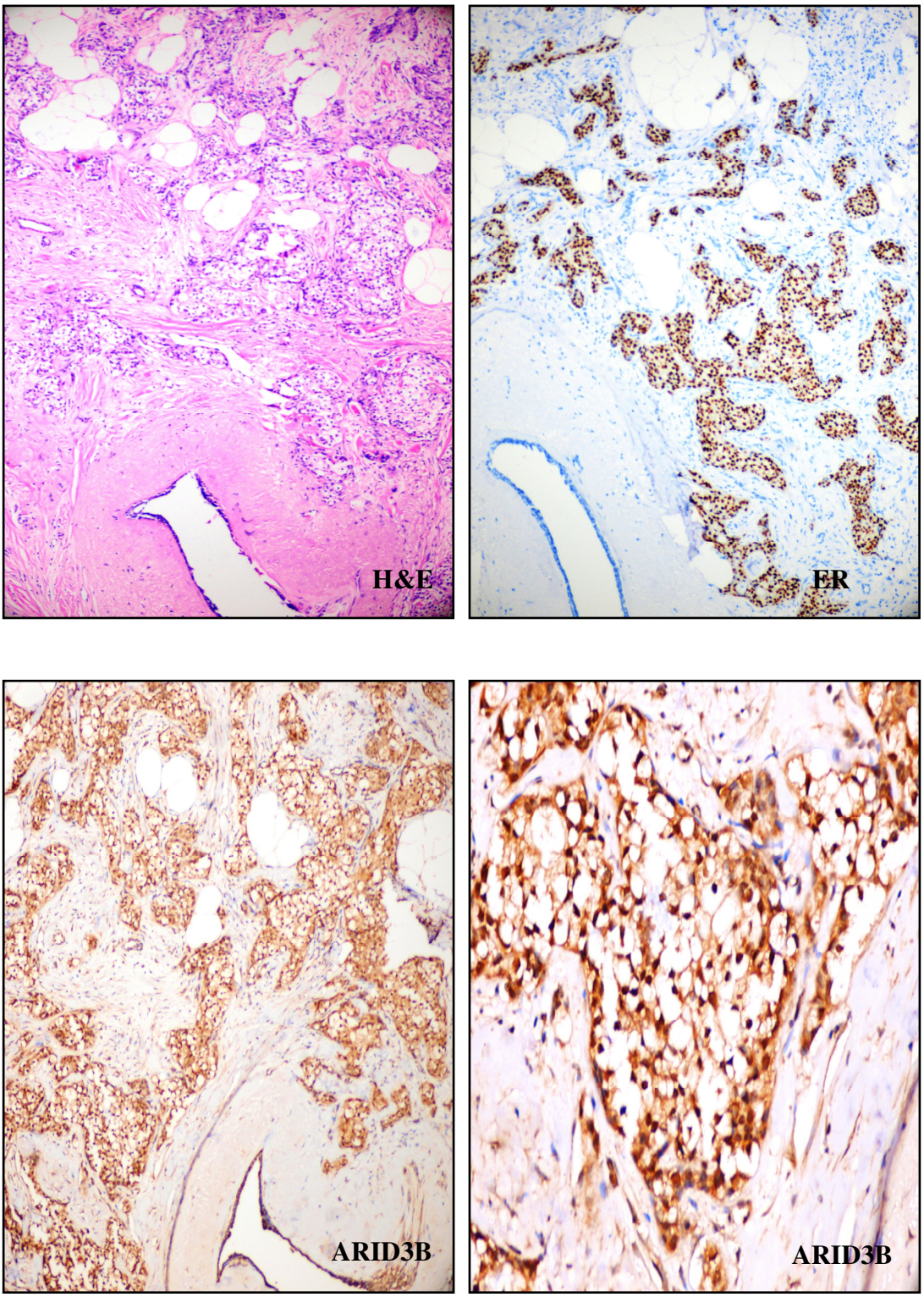


Figure 4. A malign breast cancer sample with high ER staining intensity and prevalence exhibited high nuclear ARID3B expression (H&E 10X, ER 10X, ARID3B 10X, ARID3B 40X).

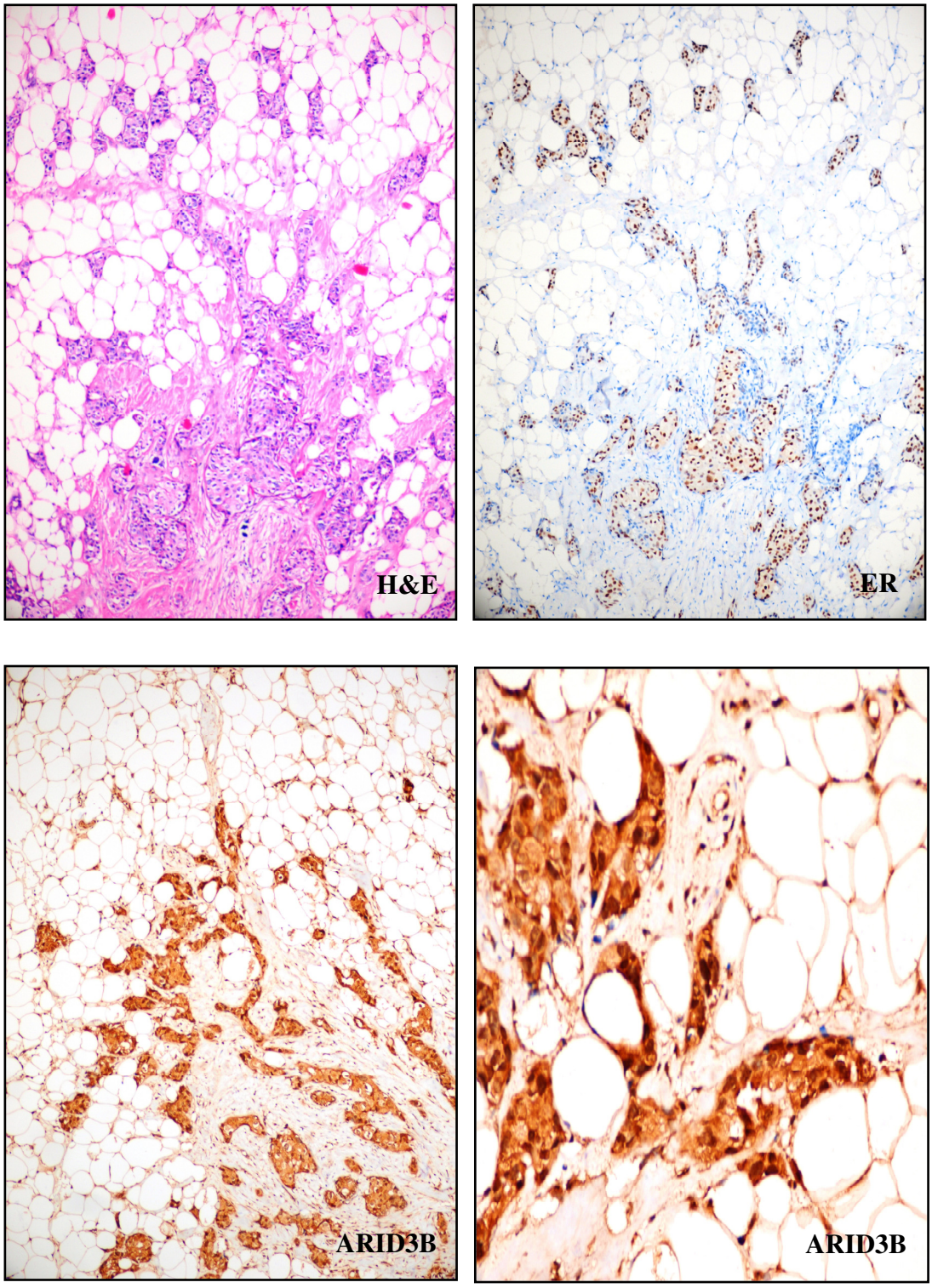


Figure 5. Malign breast tumor sample with low mitotic activity and strong ER expression exhibited strong nuclear ARID3B expression (H&E 10X, ER 10X, ARID3B 10X, ARID3B 40X).

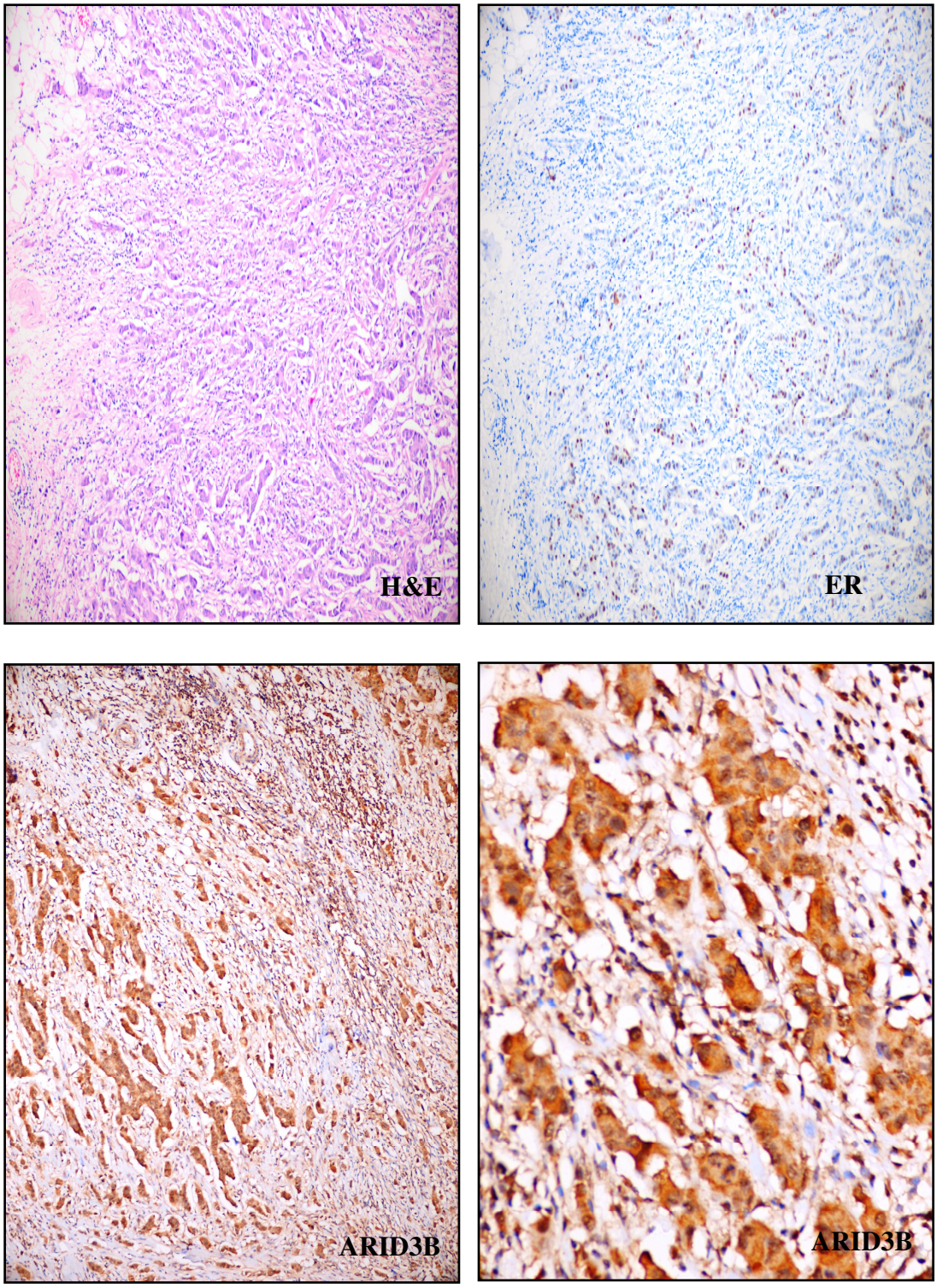


Figure 6. A malign breast tumor with moderate nuclear pleomorphism and low ER expression showed moderate ARID3B expression (H&E 10X, ER 10X, ARID3B 10X, ARID3B 40X).

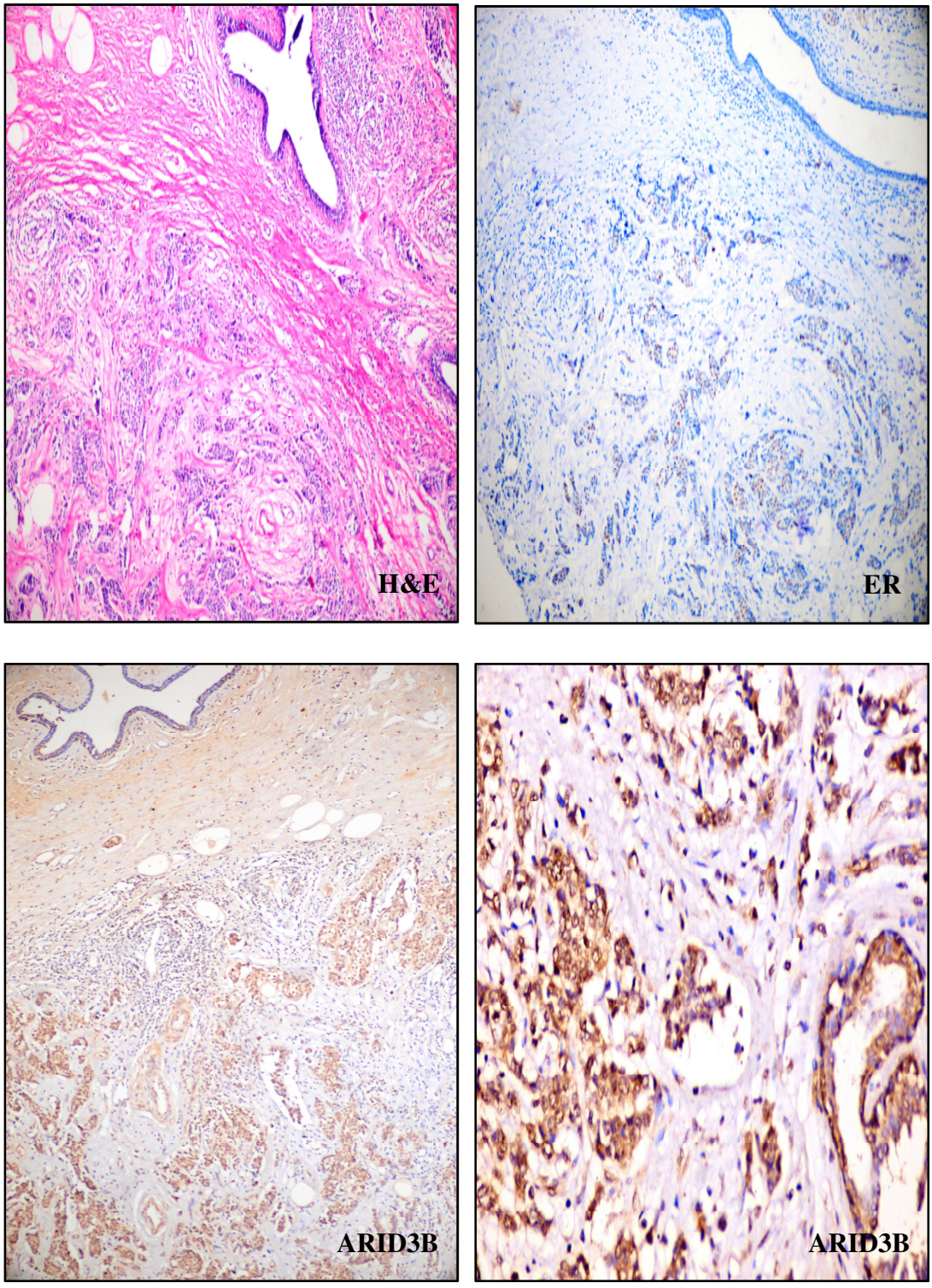


Figure 7. Malign breast tumor sample with weak ER staining intensity and prevalence and moderate ARID3B expression (H&E 10X, ER 10X, ARID3B 10X, ARID3B 40X).

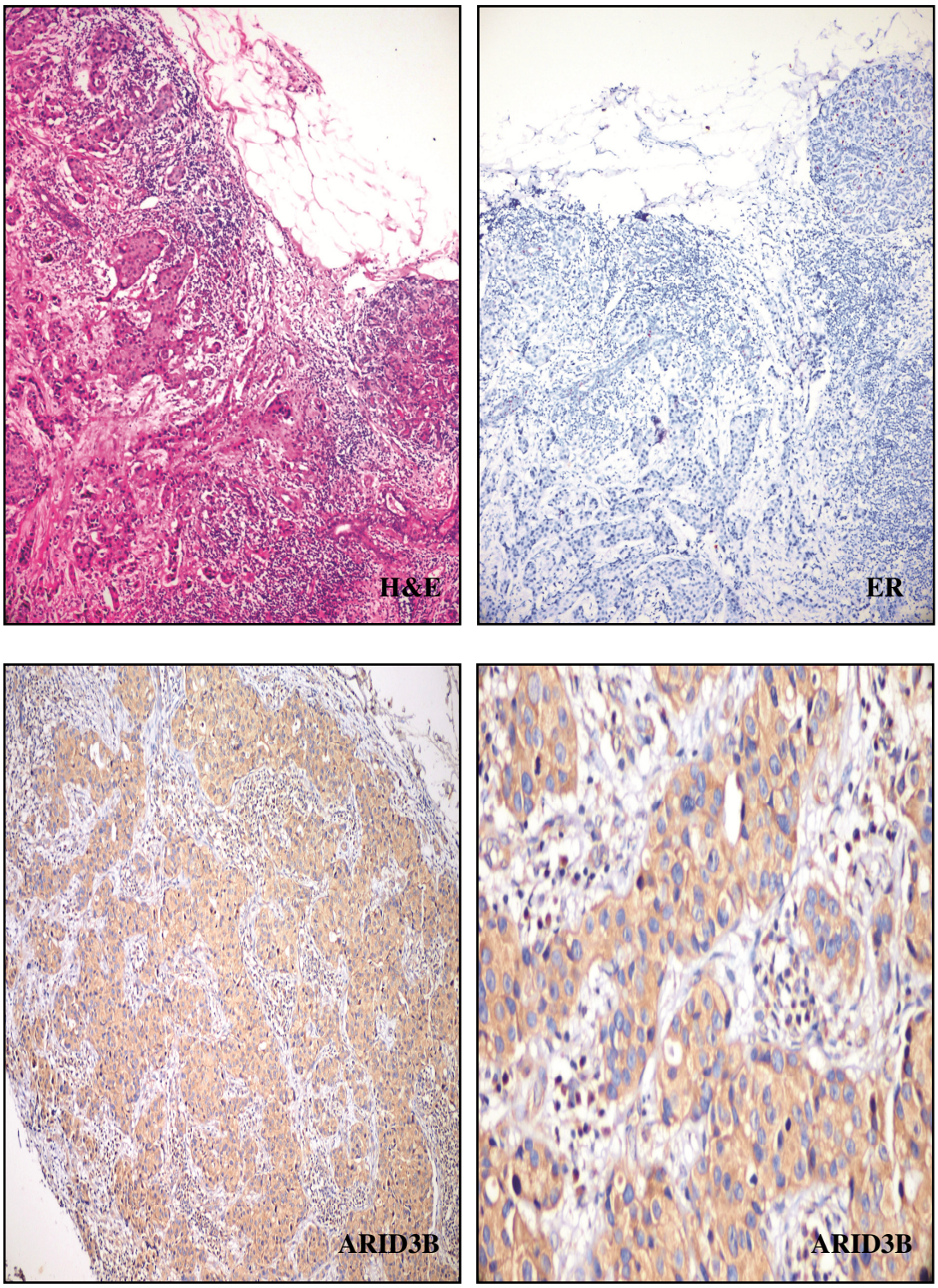


Figure 8. ER negative malign breast tumor sample with little or no evidence of glandular differentiation had no nuclear ARID3B staining. Normal breast tissue was used as a positive control for ER staining (H&E 10X, ER 10X, ARID3B 10X, ARID3B 40X).

ER positive breast tumors have better prognosis and better response to endocrine therapy than ER negative tumors (Rosai, 2011). ER related pathways and ER regulated proteins could be very important in remodeling our understanding about breast tumors etiology and its treatment. ARID3B is a novel protein and correlation between ARID3B nuclear expression and ER positivity is a novel finding.

3.5. Relation of ARID3B Expression with Tumor Grade and Mitotic Index of Tumor

When nuclear ARID3B was evaluated in terms of tumor grade and mitotic index of tumors, we detected a negative correlation.

Representative tumor samples are shown in Figure 9 to Figure 11.

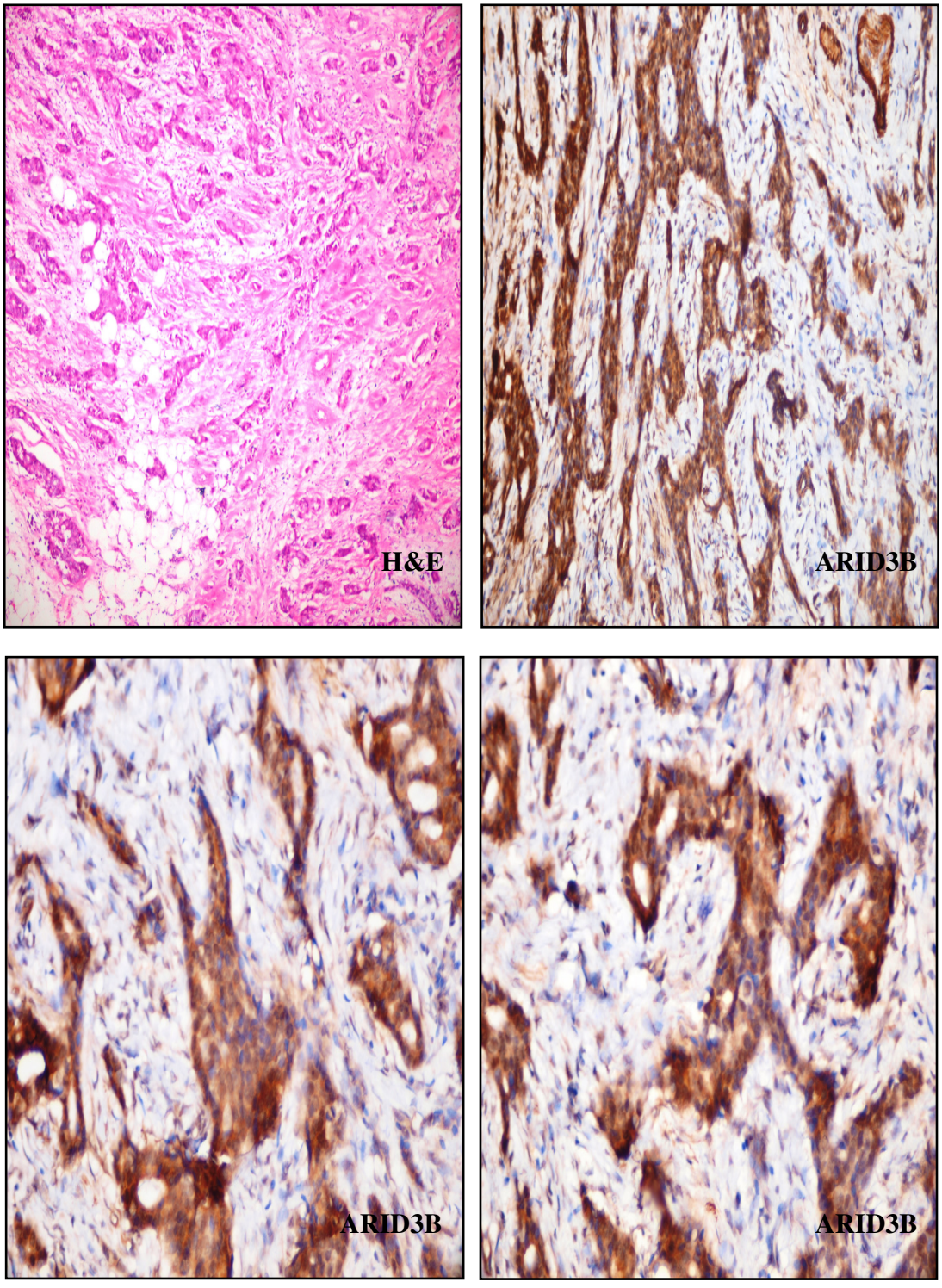


Figure 9. Grade 1 breast tumor sample with low mitotic activity and tubule formation in the majority of the tumor exhibited strong nuclear ARID3B staining intensity and prevalence (H&E 10X, ARID3B 10X, ARID3B 40X, ARID3B 40X).

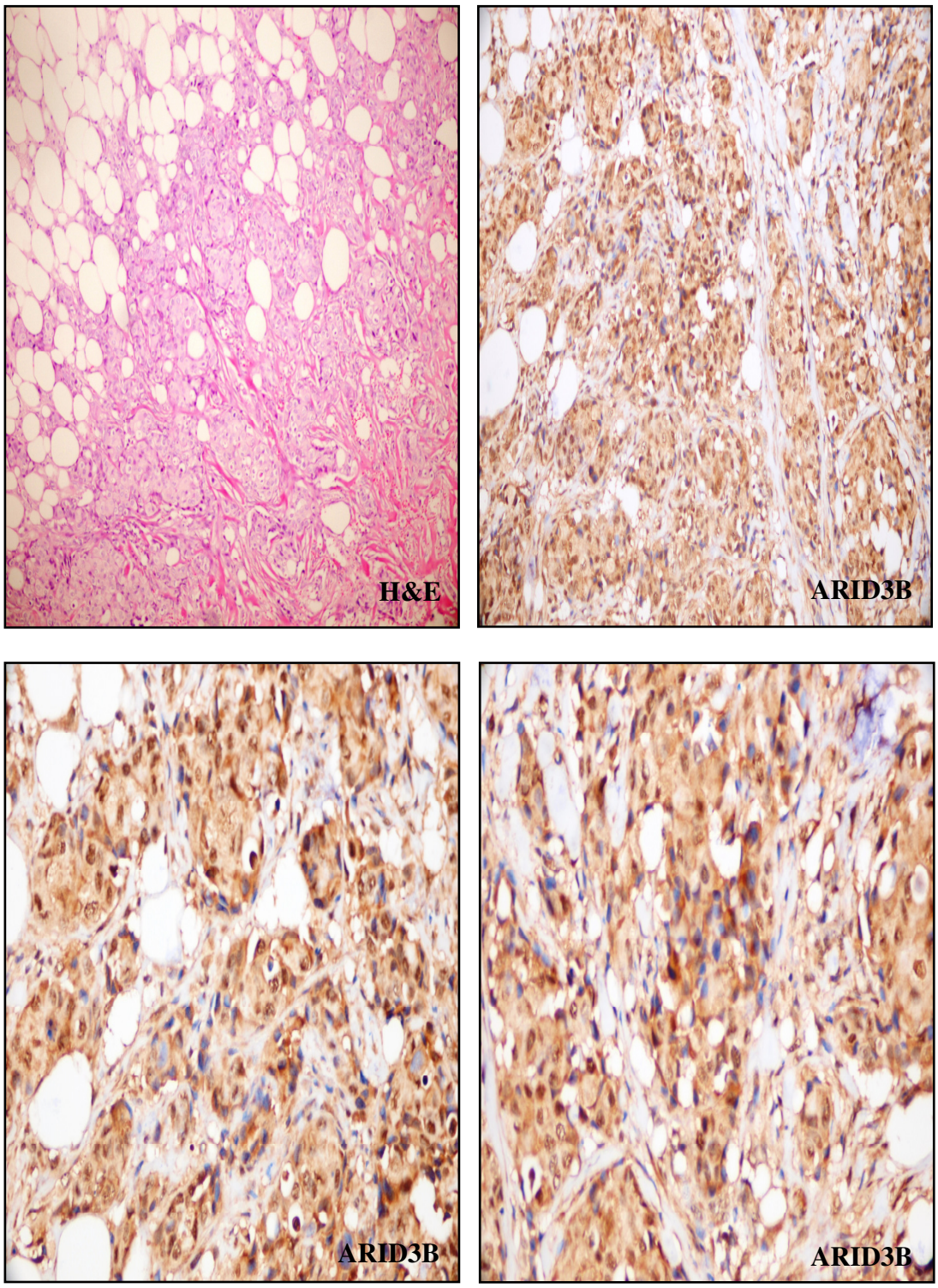


Figure 10. Grade 2 breast tumor sample with moderate mitotic activity and low level tubule formation exhibited moderate nuclear ARID3B expression (H&E 10X, ARID3B 10X, ARID3B 40X, ARID3B 40X).

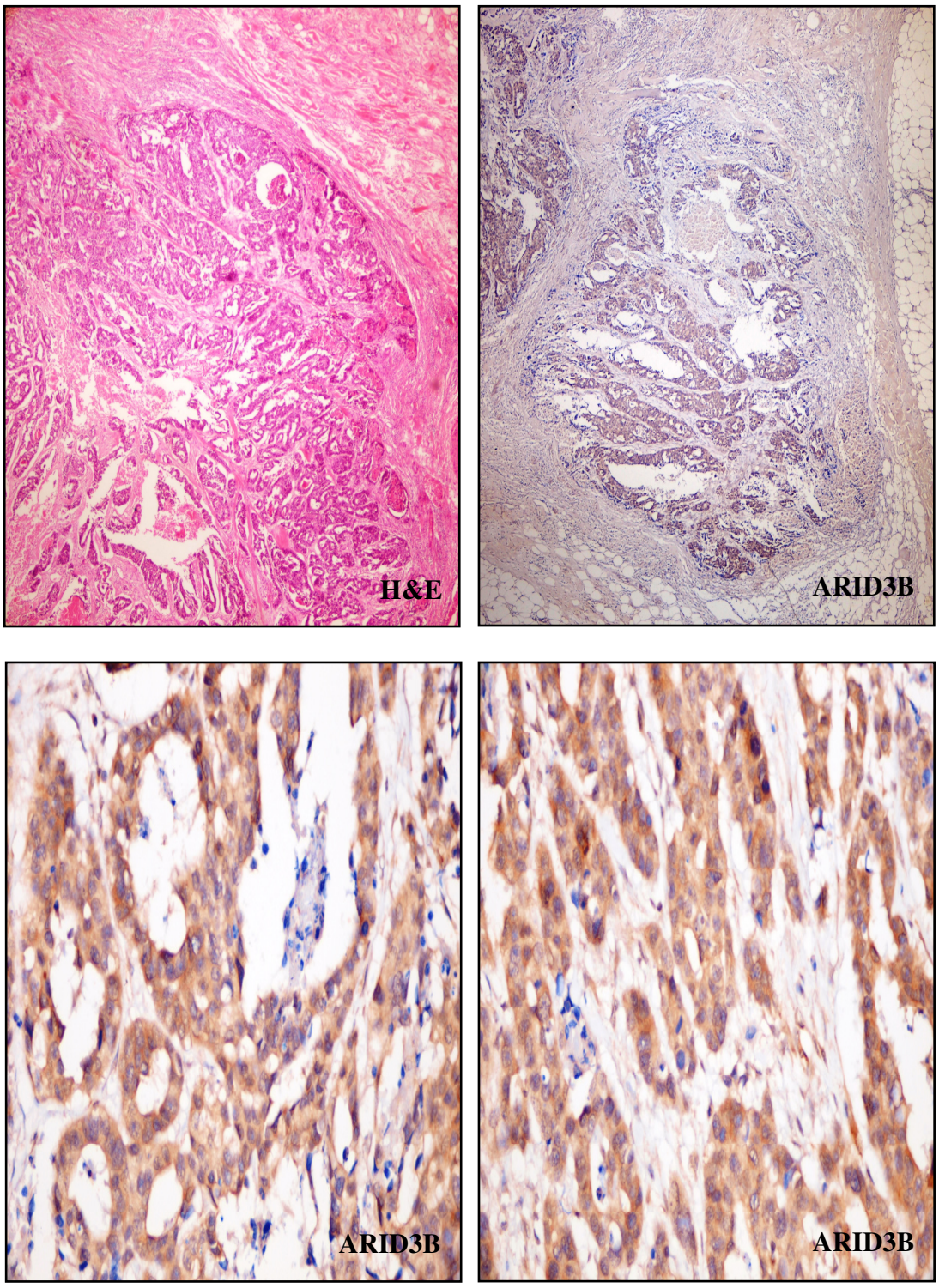


Figure 11. Grade 3 breast tumor sample with high mitotic activity and nuclear pleomorphism had no ARID3B staining (H&E 10X, ARID3B 10X, ARID3B 40X, ARID3B 40X).

ARID3B was first defined as a protein that could bind to an important cell cycle regulator RB (Retinoblastoma) (Numata et al., 1999). Our results showed ARID3B nuclear expression to be negatively correlated with mitotic index of tumors. A possible link between RB and ARID3B in breast cancers will be interesting to further evaluate in terms of cell cycle regulation, which may have an impact on mitotic index.

A number of researchers have reported that ARID3B has grade dependent expression patterns in some tumor types. Kobayashi et al suggest that grade IV neuroblastoma has increased levels of ARID3B compared with lower grades of neuroblastoma (Kobayashi et al., 2006). In another study, Dahl et al show high levels of ARID3B expression in malign serous ovarian tumors versus benign variants (Dahl et al., 2009). On the contrary, Samyesudhas et al suggest that the expression of ARID3B decreases during tumor progression in the esophagus and stomach cancers (Samyesudhas et al., 2014). The contradictory findings may be due to the fact that there are multiple isoforms recognized by IHC antibodies and unfortunately all of these isoforms are not addressed in the literature. From studies done in our lab we know that all mRNA isoforms are expressed in normal breast tissue as well as several breast cancer cell lines (Oguz Erdogan et al., 2014).

Overall, findings of the current study suggest ARID3B full length and possibly 23 kDA isoform to be negatively correlated with aggressive tumors based on ER staining and mitotic index.

3.6. Relation of ARID3B Expression with HER2 Expression of Tumor

Our IHC results also suggested nuclear ARID3B expression and HER2 status to be negatively correlated. This also means, HER2 negativity was observed in 63% (12/19) of nuclear ARID3B staining cases. Representative tumor samples are shown in Figure 12 and Figure 13.

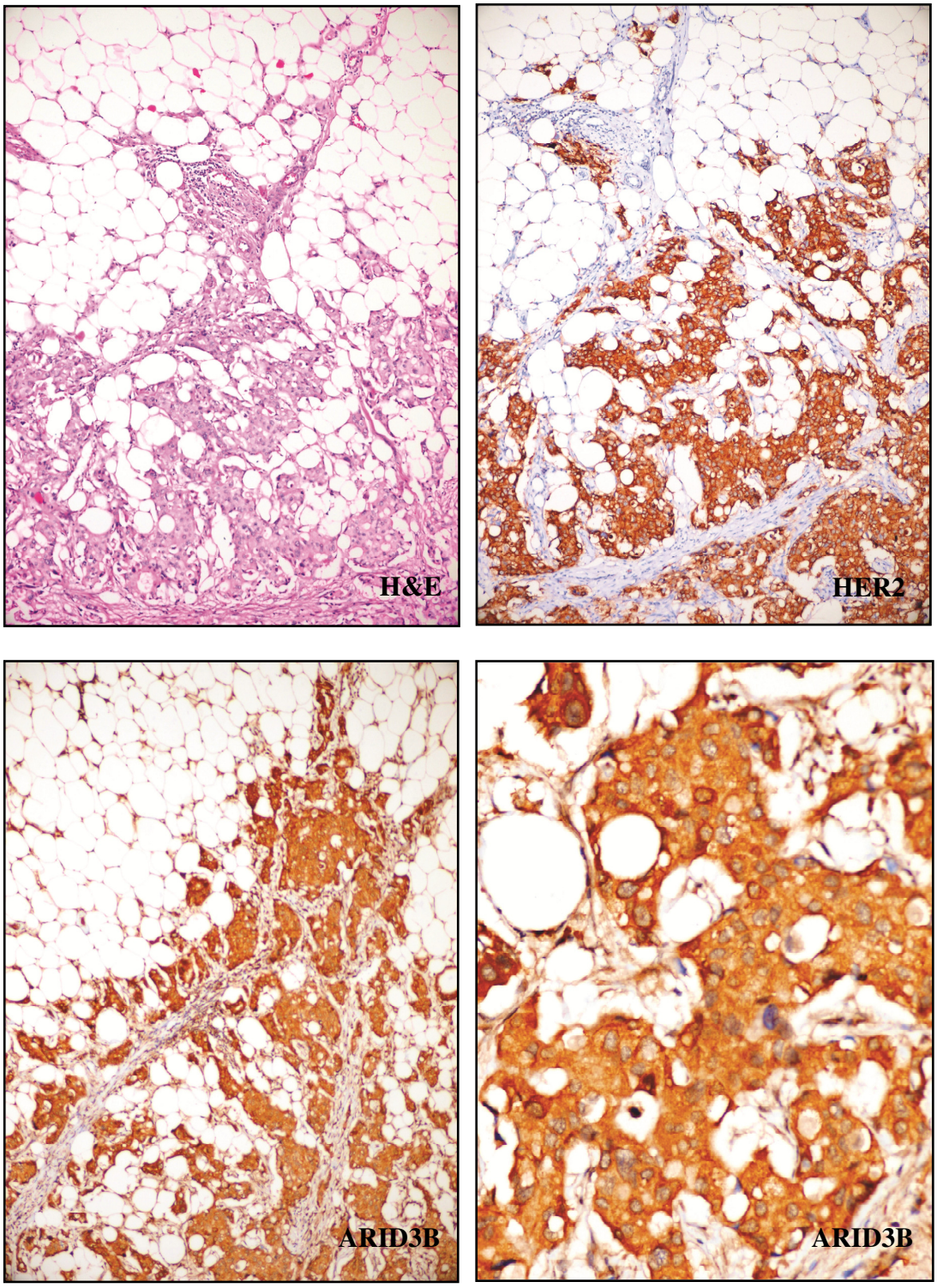


Figure 12. Malign breast tumor sample with strong and diffuse HER2 expression had no nuclear ARID3B expression (H&E 10X, HER2 10X, ARID3B 10X, ARID3B 40X).

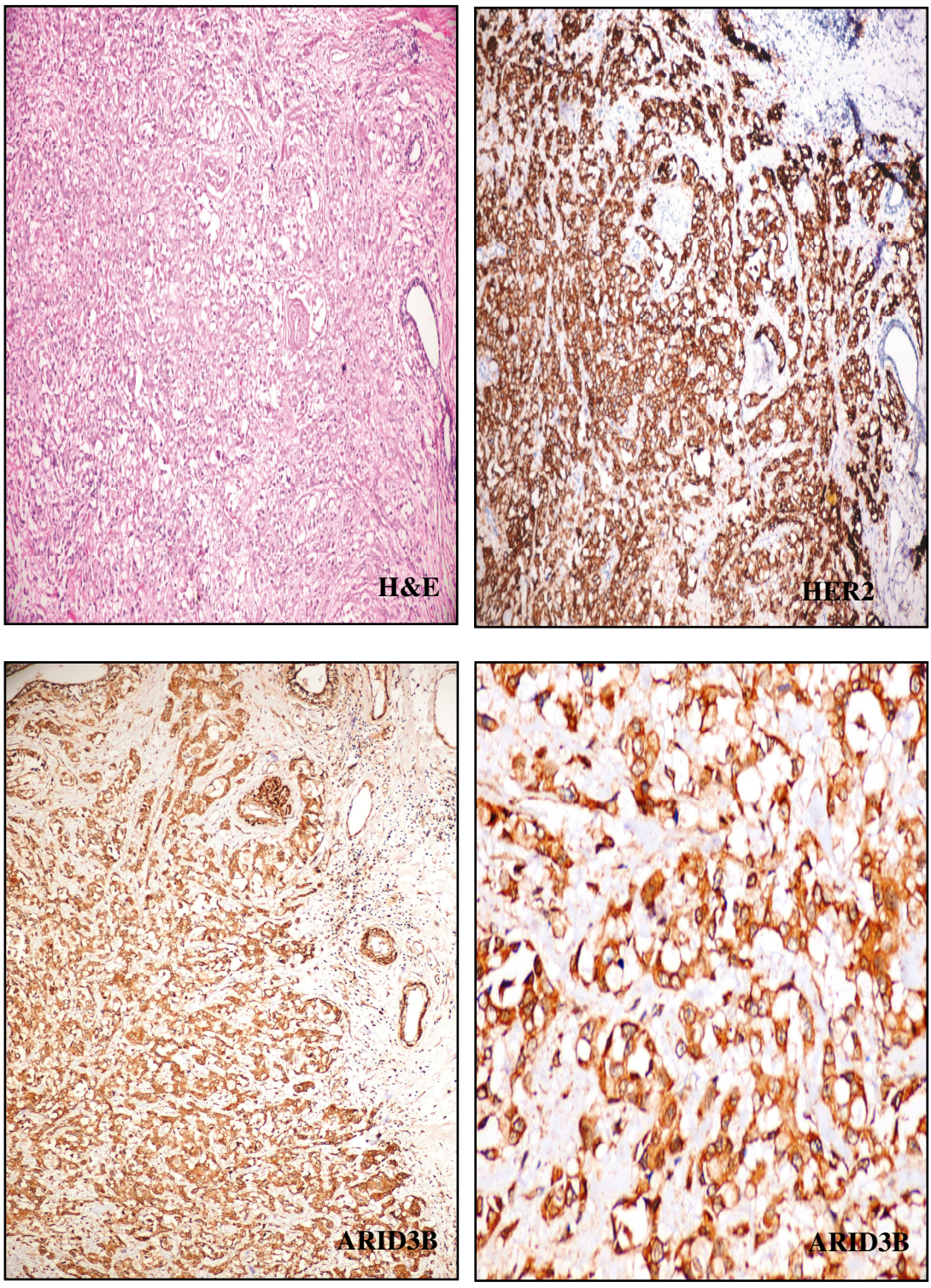


Figure 13. High grade breast tumor sample with marked variations in nuclear size, shape, HER2 staining intensity and prevalence showed very low level nuclear ARID3B staining (H&E 10X, HER2 10X, ARID3B 10X, ARID3B 40X).

Our results suggested a negative correlation with ARID3B nuclear staining and HER2 positivity. In all 12 cases (ARID3B (+), HER2 (-)), patients also had strong ER positivity. The remaining HER2 positive patients (13 cases) had negative nuclear ARID3B staining. 6 of these cases were ER positive and rest of them was ER negative.

We also went back to ER+, ARID3B+ tumors to see their HER2 levels. There were 17 tumors in this group and 12 of these tumors were HER2 negative and rest was HER2 positive (n=5). While the ER, HER2 and ARID3B expression patterns are not 100% correlative, a potential association exists. Based on these data, studies are underway to delineate a potential relationship between ARID3B, ER positivity and HER2 negativity in breast cancers.

From a study in our group, we also determined that miR-125b can target ARID3B and ARID3B was linked to motility of cells in breast cancer cell lines, independent of HER2 effect (Akhavantabasi et al., 2012). The connection between ARID3B, miR-125 family and possibly with ER and HER2 signaling pathways is quite interesting and worth investigating.

CHAPTER 4

CONCLUSION

Invasive breast cancer is the most common tumor type in women (Lakhani et al., 2012). The main prognostic parameters include tumor histologic grade, tumor stage, hormonal status of the tumor and lymphovascular invasion (Kumar et al., 2009; Lakhani et al., 2012). These prognostic factors indicate the course of the disease and guide the clinicians about right medical therapy for each patient (Lønning, 2007). Therefore, investigation of new prognostic factors, medical therapies and imaging methods can change the course of the disease and cause significant changes on patients' survival. These studies also provide very important data about molecular biology of the disease and elucidate novel genes and pathways deregulated in breast cancer.

ARID3B is a novel protein with evolutionary conserved DNA-binding domain (Wilsker et al., 2005). Previous studies have reported importance of ARID3B both on physiological and pathological processes. It has been related with mesenchymal tissue, cardiovascular system, cytoskeletal system and neuronal tissue formation during embryogenesis (Casanova et al., 2011; Samyesudhas et al., 2014; Takebe et al., 2006; Uribe et al., 2014). In addition to that, ARID3B has also been linked to many different tumor types. Studies demonstrate alterations in ARID3B expression levels in neuroblastoma and ovarian cancer (Kobayashi et al., 2006; Roy, et al., 2014). Increased levels of ARID3B is related to worse prognosis in these tumor groups (Kobayashi et al., 2006; Roy, et al., 2014). However, in esophagus and gastric malignancies expression levels of ARID3B tend to decrease with tumor progression (Samyesudhas et al., 2014).

The main goal of the current study was try to investigate the role of ARID3B in breast cancer. For this reason we examined 63 FFPE blocks of primary invasive breast carcinoma samples by using immunohistochemistry and compared the results with other prognostic factors of invasive breast cancer. One of the most significant findings to emerge from this study is positive correlation between ER expression and ARID3B nuclear expression. This study has also shown that nuclear ARID3B expression is negatively correlated with tumor grade, mitotic activity and HER2 expression of tumor cells. Taken together, these results suggest that high ARID3B expression levels in primary breast cancer may be related with a better prognosis and long survival.

Different expression levels of ARID3B in different cancer types were suggested in many studies. Gastric cancers and esophagus cancers show low levels of ARID3B expression, however neuroblastoma and ovarian cancers show high expression levels of ARID3B (Samyesudhas et al., 2014). This difference of expression patterns of ARID3B may be due to tissue dependent function or role of ARID3B and its isoforms.

This was the first study reporting a link between ARID3B expression and major prognostic factors of breast cancer.

For future studies, delineating the ARID3B isoform functions may be crucial to further understand and study the function of ARID3B in breast and other cancers. This study was significant in terms of revealing a potentially important axis involving ER, ARID3B and HER2 expression in breast cancers. Therefore future studies in our laboratory are focused on understanding the mechanistic relationship between these proteins.

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