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Original contribution

PD-L1 and CD8 are associated with deficient mismatch repair status in triple-negative and HER2-positive breast cancers[☆,☆☆,★]

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Summary Triple-negative and HER2-positive breast cancers (BCs) are more aggressive than hormone receptor–positive/HER2-negative BCs and show higher levels of tumor-infiltrating lymphocytes (TILs) and PD-L1 expression. Recently, US Food and Drug Administration approved anti–PD-L1 immunotherapy for solid tumors with deficient mismatch repair (MMR). In this study, we aimed to examine the prevalence of deficient MMR and its association with checkpoint immune markers in BCs. Immunohistochemistries (IHCs) with anti–MMR proteins (MLH1, PMS2, MSH2 and MSH6) and multiplex IHCs with anti–PD-L1, anti-CD8 or anti-CD163 were performed on tissue microarrays (TMAs) with 101 triple-negative BCs (TNBC) and 197 HER2-positive BCs. Additional IHCs for MMR proteins were also performed on whole-tissue sections from selected cases. Thirteen cases (4.4%) showed complete loss of MMR protein on TMAs, including 7 TNBCs (6.9%) and 6 HER2-positive BCs. On whole-tissue sections, only one of 13 cases showed complete loss of MMR proteins, while the other 12 cases showed partial loss. PD-L1 expression was identified in 37% of cases and was significantly higher in TNBCs than in HER2-positive BCs (71% versus 19%). Furthermore, BCs with complete/partial loss of MMR demonstrated significantly more PD-L1 and CD8 expressions than BCs with preserved MMR proteins. Although complete loss of MMR proteins exists in an extremely low frequency, partial loss is not uncommon in BCs. The association of partial loss of MMR proteins with increased PD-L1 and CD8 expression suggests a potential use of MMR testing as a screening method for anti–PD-L1 immunotherapy in BCs.

1. Introduction

Germline alterations in four key human proteins in the DNA mismatch repair (MMR) pathway—MLH1, PMS2, MSH2, and MSH6—were identified to be responsible for Lynch syndrome [1,2]. Their sporadic somatic mutations have also been found in tumors unrelated to hereditary syndromes, mostly colorectal cancer and endometrial cancer [3-5]. Cancers with deficient MMR (dMMR) are predicted to have a large number of mutation-associated neoantigens that might be recognized by the immune system, and then the cancers cells are killed by cytotoxic T cells [6]. Checkpoint immune molecules PD1 and PD-L1 can limit this antitumor immune activity by inhibiting cytotoxic T cell function [7-9]. Based on a recent study, significant portion of colorectal cancer patients with dMMR have achieved excellent and durable responses to

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PD1/PD-L1 blockage immunotherapy [10]. Similar efficacy results were demonstrated in patients with advanced dMMR cancers across 12 tissue types including breast cancer (BC) [11].

Unlike in colorectal and endometrial cancer [12-14], very few studies have investigated dMMR and the association between dMMR and PD-L1 expression or other checkpoint immune markers in BCs [15,16]. Triple-negative breast cancers (TNBCs) and HER2-positive BCs are more aggressive than hormone receptor–positive/HER2-negative BCs and show higher levels of tumor-infiltrating lymphocytes (TILs) and PD-L1 expression [17-21]. In this study, we investigated MMR status and its association with PD-L1 and checkpoint immune markers in TNBCs and HER2-positive BCs.

2. Materials and methods

2.1. Patients and specimens

This study was approved by the Ohio State University Institutional Review Board. The cohort included 216 HER2-positive and 119 triple-negative breast carcinoma cases. Clinical and pathological characteristics, including patient's age, tumor grade, tumor size, lymph node status and clinical outcome were collected.

Estrogen receptor (ER) and progesterone receptor (PR) were determined by standard immunohistochemistry (IHC) using anti-ER antibody (clone 1D5 or SP1, Dako, Carpinteria, CA or Spring Bioscience, Pleasanton, CA) and anti-PR antibody (clone PgR 636, Dako, Dako, Carpinteria, CA). HER2 status was determined by HER2 IHC (clone 4B5, Ventana, Tucson, AZ) and/or HER2 fluorescence in situ hybridization (FISH). The FISH analysis with CEP17 probe was performed using the dual-color Vysis FDA-approved PathVysion *HER2* DNA Probe Kit (Abbott Molecular, Des Plaines, IL). The signals for *HER2* gene and CEP17 were visualized under a fluorescence microscope using appropriate filters. The average numbers of *HER2* and CEP17 signals per cell were recorded for at least 50 cells, and the *HER2:*CEP17 ratio was calculated for each case. The HER2 IHC results were interpreted by subspecialist breast pathologists and HER2 FISH results were interpreted by specialist molecular pathologists.

2.2. Tissue microarrays

A tumor block representative of primary resected tumor specimen was collected from each case through Tissue Archive Services. A pair of tissue microarrays (TMAs) representative of each tumor was constructed with core size of 1.5 mm at our pathology core facility.

2.3. MMR protein immunohistochemistry

MMR proteins were assessed by IHCs for MLH1 (NovoCastra, clone: ES05), PMS2 (BD, clone: A16–4), MSH2 (Calbiochem, clone: FE11), and MSH6 (Epitomics, clone: EP 49) on TMA sections and whole-tissue sections of selected cases using clinically-validated Bond (MLH1, MSH6) and Dako (MSH2, PMS2) immunostainers, each at a dilution of 1/200 and with colon cancer as control tissue. The positive control for all four mismatch repair protein IHCs was a colon cancer specimen with retained all four mismatch repair proteins. The negative controls were different colon cancer specimens with complete loss for each mismatch repair protein. IHC for any of the four MMR proteins was considered positive if definite nuclear staining was detected in carcinoma cells. MMR deficiency tumors were defined as the complete loss of nuclear staining in any of those four proteins. Any heterogenous staining pattern with at least 10% of loss of mismatch repair proteins was defined as partial loss of mismatch repair proteins. These heterogenous staining patterns included regional and intermixed patterns.

2.4. Multi-color multiplex immunohistochemistry and assessment of checkpoint immune system

Multi-color multiplex immunohistochemical assay using anti–PD-L1 (clone SP263, rabbit, Ventana), anti-CD8 (clone MRQ26, mouse, Ventana) and anti-CD163 (clone SP57, rabbit, Ventana) were performed on TMA sections according to manufacturers' protocol. The immunohistochemistry was evaluated with consensus viewing by two pathologists (YH and ZL). A positive PD-L1 expression was defined as any membranous staining in $\geq$1% of tumor cells or immune cells. PD-L1 expression was assessed in both tumor cells (PD-L1 TC) and immune cells (PD-L1 IC). Tumoral CD8 (IT-CD8+) was scored semi-quantitatively as a percentage of the tumoral area infiltrated with CD8+ cells to the entire tumoral area. Peritumoral CD8 (PT-CD8+) was scored semiquantitatively as a percentage of the peritumoral stromal area with CD8+ cell infiltrates to the entire peritumoral stromal area. The method is based on the recommendation from international tumor-infiltrating lymphocytes working group [22]. The cut-off percentage for CD8+ cells was 10%.

2.5. Statistical analyses

All data were analyzed using SAS version 9.4 for Windows (SAS Institute, Cary, NC). The relationship between MMR status, checkpoint immune system and clinicopathological features was assessed using Fisher's exact test or χ^2 test. $P <. 05$ was considered statistically significant.

3. Results

3.1. Clinical and pathological characteristics of study cohort

A total of 216 surgically resected primary HER2-positive BCs and 119 TNBCs were included in the study. There

Table 1 Clinical and pathological characteristics of 216 HER2-positive BCs and 119 TNBCs

Total case #	HER2+		TNBC		*P*
	216		119		
Age (y) (median, range)	53	27–88	52	28–79	NS
Race					
African-American	14	6.4%	15	12.6%	.033
White	191	88.4%	101	84.9%	NS
Other	11	5.1%	3	2.5%	NS
Phenotype					
Ductal	200	92.6%	114	95.8%	NS
Lobular	7	3.2%	0	0.0%	
Mixed *	1	0.5%	1	0.8%	
Other **	8	3.7%	4	3.4%	
Nottingham grade					
3	135	62.5%	105	88.2%	<.001
<3	79	36.6%	13	10.9%	
Unknown	2	0.9%	1	0.8%	
Hormone receptor					
ER+	126	58.3%	0	0.0%	<.001
PR+	99	45.8%	0	0.0%	<.001
T stage					
1	116	53.7%	53	44.5%	NS
2	83	38.4%	57	47.9%	
3	13	6.0%	6	5.0%	
4	4	1.9%	3	2.5%	
Lymph node					
Positive	102	47.2%	47	40.9%	NS

Abbreviations: TNBC, triple-negative breast carcinoma; ER, estrogen receptor; PR, progesterone receptor; NS, not significant.

* Mixed phenotype indicated a mixed ductal and lobular phenotype.

** Other phenotypes included apocrine carcinoma, metaplastic carcinoma and carcinoma with medullary features.

was no significant difference in age, T stage and lymph node status between HER2-positive BCs and TNBCs, but there were significantly more African-Americans (12.6% versus 6.4%), more Nottingham grade 3 (88.2% versus 62.5%) in TNBCs than in HER2-positive BCs (Table 1).

Table 2 MMR status and checkpoint immune markers of 197 HER2-positive BCs and 101 TNBCs

Total case #	Total		HER2+		TNBC		*P*
	298		197		101		
PD-L1							
All PD-L1	109	36.6%	33	16.8%	76	75.2%	<.001
PD-L1 TC	22	7.4%	11	5.6%	11	10.9%	.056
PD-L1 IC only	87	29.2%	22	11.2%	65	64.4%	<.001
Immune markers							
IT-CD8+	92	30.9%	44	22.3%	48	47.5%	<.001
PT-CD8+	144	48.3%	62	31.5%	82	81.2%	<.001
MMR on TMAs							
MLH1	8	2.7%	2	1.0%	6	5.9%	.011
PMS2	10	3.4%	3	1.5%	7	6.9%	.012
MSH2	0	0.0%	0	0.0%	0	0.0%	NS
MSH6	3	1.0%	3	1.5%	0	0.0%	NS
dMMR	13	4.4%	6	3.0%	7	6.9%	NS

Abbreviations: TNBC, triple-negative breast carcinoma; PD-L1 TC, PD-L1 expression in tumor cells; PD-L1 IC, PD-L1 expression in immune cells, IT-CD8+, intratumoral CD-positive cells; PT-CD8+, peritumoral CD8-positive cells; MMR, mismatch repair proteins; dMMR, deficient mismatch repair; TMA, tissue microarray; NS, not significant.

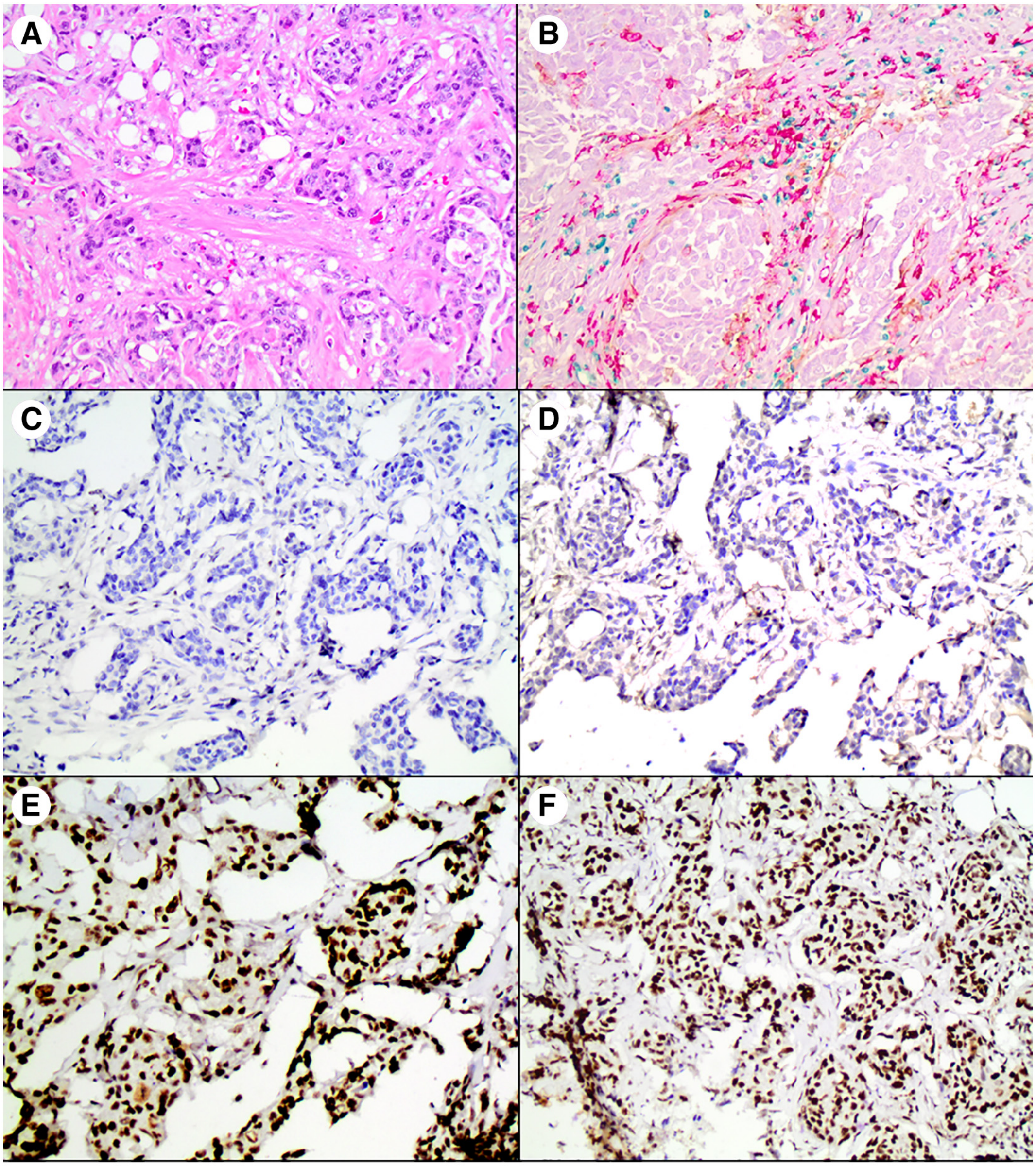

Fig. 1 One TNBC case with loss of MLH1 and PMS2 on both TMA and whole-tissue section. A, H&E. B, Multiplex IHCs with PD-L1 staining (brown), CD8 (green) and CD163 (red) show weak PD-L1 staining in stromal cells, lymphocytes and histiocytes. C, IHC of MLH1 shows loss of nuclear staining. D, IHC of PMS2 shows loss of nuclear staining. E, IHC of MSH2 shows retained nuclear staining. F, IHC of MSH6 shows retained nuclear staining; 200×.

3.2. Assessment of checkpoint immune markers (PD-L1 and CD8) and MMR proteins (MLH1, PMS2, MSH2 and MSH6) in HER2-positive BC (n = 197) and TNBC (n = 101) TMAs

Among all TMA cases, checkpoint immune markers and MMR protein IHCs were successfully performed for 197 HER2+ BCs and 101 TNBCs. PD-L1was identified in 109 cases (36.6%) including 22 cases (7.4%) with PD-L1 expressing tumor cells and 87 cases (29.2%) with PD-L1 expressing immune cells only. PD-L1 was expressed in immune cells more frequently in TNBCs than in HER2+ BCs, but not in tumor cells (Table 2).

On TMAs, 13 cases (4.4%) showed dMMR, including 6 HER2-positive BCs (3%) and 7 TNBCs (6.9%). All 13 cases were invasive ductal carcinoma. Eight cases had loss of MLH1 and PMS2, 2 had PMS2 loss, and 3 had MSH6 loss. However, only one TNBC case with loss of MLH1 and PMS2 on TMA also showed complete loss of those two proteins on whole-tissue section (Fig. 1), while the other 12 cases only showed partial loss of MMR proteins (heterogenous expression) on whole-tissue sections (Table 2 and Fig. 2). The patient with complete loss of MLH1 and PMS2 had a previous breast cancer on the other side diagnosed and treated at an age younger than 50. However, the detailed information and the specimen from her previous breast cancer were not available.

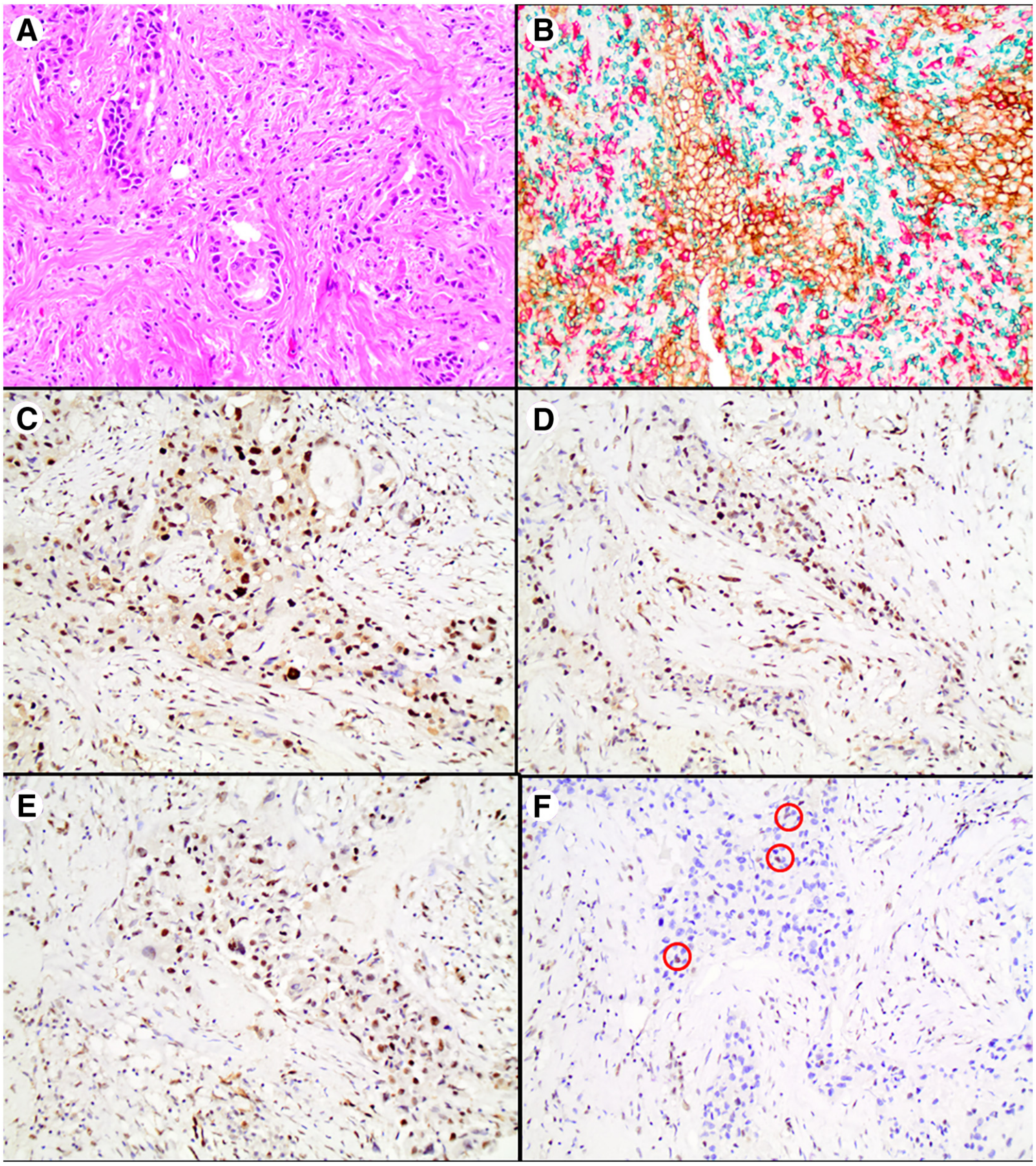

Fig. 2 One HER2+ BC case with loss of MSH6 on TMA, but partial loss of MSH6 on whole-tissue section. A, H&E. B, Multiplex IHCs with PD-L1 staining (brown), CD8 (green) and CD163 (red) show strong PD-L1 staining in tumor cells, extensive lymphocytes and histiocytes. C, IHC of MLH1 shows retained nuclear staining. D, IHC of PMS2 shows retained nuclear staining. E, IHC of MSH2 shows retained nuclear staining. F, IHC of MSH6 shows retained nuclear staining only in rare circled tumor nuclei, but loss of staining in the majority of tumor nuclei; 200×.

She also had an endometrial curettage with complex atypical hyperplasia before, but the treatment information and final pathology were not available. Her family history included breast cancer and other cancers in family members.

3.3. Association of checkpoint immune markers and MMR status

Next, we examined the association between dMMR and checkpoint immune markers (PD-L1 and CD8). Patients with complete/partial loss of MMR (n = 13) were significantly younger and demonstrated more PD-L1 and CD8 expressions than patients with total preservation of MMR proteins (n = 285) (Table 3).

3.4. MMR status in 30 clinical primary and metastatic breast carcinomas

In addition, MMR protein IHCs were performed on whole sections from 30 clinical breast carcinomas, including 28 metastatic carcinoma specimens and two primary breast carcinoma specimens. Five were HER2+ BCs and 25 were TNBCs. No MMR protein loss including partial loss was detected in any of these clinical cases (Table 4).

Table 3 The comparison of PD-L1 and CD8 expression between 13 cases with complete/partial loss of MMR proteins and 285 cases with normal MMR protein expression

Case #	Total		dMMR		Normal MMR		*P*
	#	%	#	%	#	%	
	298		13		285		
Age (y)	53.9	27–88	45.5	33–58	54.3	27–88	.046
Nottingham grade 3	221	74.2%	11	84.6%	210	73.7%	NS
T stage (>1)	149	50.0%	7	53.8%	142	49.8%	NS
Lymph node (+)	139	46.6%	6	46.2%	133	46.7%	NS
Any PD-L1+	109	37%	9	69%	100	35%	.012
PD-L1 TC	22	7%	1	8%	21	7%	NS
PD-L1 IC only	87	29%	8	62%	79	28%	.011
IT-CD8+	92	31%	8	62%	84	29%	.014
PT-CD8+	144	48%	11	85%	133	47%	.007

Abbreviations: PD-L1 TC, PD-L1 expression in tumor cells; PD-L1 IC, PD-L1 expression in immune cells, IT-CD8+, intratumoral CD-positive cells; PT-CD8+, peritumoral CD8-positive cells; MMR, mismatch repair proteins; dMMR, deficient mismatch repair; TMA, tissue microarray; NS, not significant.

Table 4 Mismatch repair protein IHC results of additional 30 clinical primary and metastatic breast carcinomas

	HER2+		TNBC	
Total case #	5		25	
Age (y) (median, range)	56.7	37–77	55.4	39–81
Race				
African-American	0	0%	2	8%
White	5	100%	23	92%
Location				
Primary	0	0%	2	8.0%
Metastatic	5	100%	23	92%
Hormone receptor				
ER+	3	60%	0	0%
ER–	2	40%	25	100%
PR+	1	20%	0	0%
PR–	4	80%	25	100%
MMR status				
MLH1 loss	0	0%	0	0%
PMS2 loss	0	0%	0	0%
MSH2 loss	0	0%	0	0%
MSH6 loss	0	0%	0	0%
MMR preserved	5	100%	25	100%

Abbreviations: TNBC, triple-negative breast carcinoma; MMR, mismatch repair proteins; ER, estrogen receptor; PR, progesterone receptor.

4. Discussion

Although PD-L1 expression has been successfully used a biomarker for anti–PD1/PD-L1 targeted immunotherapy in several tumors such as non-small cell lung carcinoma and melanoma [23-28], the data on its value in breast cancer has been mixed [29-31]. Recently, FDA-approved Pembrolizumab for all solid tumors with dMMR based on promising studies which demonstrated an objective clinical response to anti-PD1 immunotherapy in dMMR solid tumors [10,11]. The efficacy of anti-PD1 immunotherapy is thought to be mediated through cytotoxic T-cell function against tumor neoantigens existing in these tumors as a consequence of excessive somatic mutations caused by impaired mismatch repair function [32].

MMR deficiency is extremely rare in breast cancer with less than 1% of all breast cancers and 1.8% of triple-negative breast carcinomas [15,16,33], arguing its value as a biomarker for immunotherapy in BCs. Indeed, our experimental data have also demonstrated that complete loss of MMR protein is extremely rare, occurring in only one out of 298 tested HER2+ BCs or TNBCs. This is further confirmed by the finding that none of 30 clinical BCs tested with MMR protein IHCs showed complete loss of protein expression (Table 4). However, partial loss (heterogenous expression) of MMR proteins was observed in a considerable number of BCs in current series (~4%). Heterogenous expression (partial loss) of MMR proteins has been demonstrated before [34-36] and was also evidenced in current study with 12 cases which showed complete loss on TMAs but focal staining on whole sections. Three patterns of heterogenous staining have been described, including "intraglandular" loss (retained/lost staining within glands), "clonal" loss (distinct groups of glands/whole glands without staining), and "compartmental" loss (retained/lost staining between different blocks) [34,37]. The mechanism causing partial loss of MMR proteins include methylation and somatic events [35]. The association with checkpoint immune response and clinical significance of partial loss MMR proteins are unclear. One previous study showed PD-L1 was entirely negative in 2 BC cases with partial loss of MMR proteins [16]. However, BCs with partial loss of MMR proteins in our series demonstrated significantly more CD8+ lymphocytes and PD-L1 expression in immune cells, suggesting that BCs with partial loss of MMR proteins may benefits from anti–PD-L1 immunotherapy, although future clinical trials are warranted.

The use of TMAs posed the greatest limitation in this study, which might result in false-negative results considering heterogenous expression of PD-L1 and MMR proteins. Indeed, 12 of 13 cases with loss of MMR proteins demonstrated expression of MMR proteins on the following IHCs on whole-tissue sections. Similarly, PD-L1 can be expressed variably within and around tumors, and thus TMAs may not be representative. The other limitation is a single PD-L1 antibody clone (SP263; Roche Ventana) used in current study. Given the existence of multiple anti–PD-L1 antibodies and different cut-off values among these antibodies, it will be more informative by examining PD-L1 expression with different anti–PD-L1 antibodies. The strengths of current study include its large sample size (298 cases) and the focus of HER2+ BCs and TNBCs.

In conclusion, our data confirm that complete loss of MMR proteins is extremely rare in breast carcinoma, even in aggressive HER2+ BCs and TNBCs. However, partial loss of MMR proteins was detected in a considerable percentage of HER2+ BCs or TNBCs (4.4%). More importantly, BC with partial loss of MMR proteins demonstrated significantly more immune reaction and PD-L1 expression, suggesting these BCs may benefits from anti–PD-L1 targeted immunotherapy.

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