Virchows Archiv (2019) 475:223–231
https://doi.org/10.1007/s00428-019-02591-z

ORIGINAL ARTICLE

Immunohistochemical expression of mismatch repair proteins (MSH2, MSH6, MLH1, and PMS2) in prostate cancer: correlation with grade groups (WHO 2016) and ERG and PTEN status

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Received: 27 December 2018 / Revised: 5 April 2019 / Accepted: 3 June 2019 / Published online: 17 June 2019

Abstract

The role of DNA MMR genes in prostate cancer (PrCa) is controversial, as genetic alterations leading to microsatellite instability are incompletely defined in these tumors. *ERG* rearrangements and *PTEN* loss are concomitant events in PrCa. The aim of this study has been to analyze the immunohistochemical (IHC) expression of MSH2, MSH6, MLH1, PMS2, ERG, and PTEN and their potential association with the grade group (GG) grading system (WHO 2016) and PSA recurrence in a series of 200 PrCa (PSMAR-Biobank, Barcelona, Spain). MSH2, MLH1, PMS2, and PTEN losses were documented in 8%, 5%, 2%, and 36.5%, respectively. ERG expression was found in 48%. MSH6 showed an increase of expression with respect to basal levels in 42.1% of the cases. A statistical association between MSH6 overexpression and GG5 was found ($p = 0.0281$). ERG-*wild-type* cases were associated with single MSH2 loss ($p = 0.024$), and MSH2 and/or MLH1 loss ($p = 0.019$). The percentage of cases with PTEN loss was 20.5% (8/39) in GG1, 37.6% (53/141) of clustered GG2 to 4, and 60% (12/20) of GG5 (chi-square test, $p = 0.01$). Thus, PTEN expression loss was statistically more frequent in the upper-grade tumors. PMS2 loss was an infrequent event, but it was statistically associated with shorter time to PSA recurrence ($p = 0.011$). These results suggest the existence of an alternative non-*ERG* pathway associated with MSH2 or MLH1 expression loss. MSH6 overexpression could be a marker of aggressiveness in PrCa. The IHC assessment of DNA MMR proteins, ERG and PTEN, could identify different altered PrCa pathways, which could aid patient stratification.

Keywords Mismatch repair genes · Grade groups · ERG · PTEN · Prostate cancer

Introduction

Mismatch repair (MMR) is an excision-resynthesis system that acts as a DNA damage sensor, correcting mismatches generated during DNA replication. The best known complexes are MutSα and MutLα, formed by MSH2 and MSH6, and MLH1 and PMS2, respectively [1–4]. Defects in DNA MMR proteins are permissive for carcinogenesis, giving rise to microsatellite instability (MSI) and conferring a hypermutated status [5]. The role of MMR genes and its proteins has been extensively studied in colorectal [6] and endometrial cancer [7]. There are recent studies on MMR protein expression in prostate tumors [8–16], but the biologic and clinical meaning in this setting is not fully understood. Pritchard et al. [5] described a hypermutated microsatellite-unstable form of advanced prostate cancer (PrCa), associated with *MSH2* and *MSH6* structural rearrangements. In contrast

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to most studies in which loss of MMR proteins expression is associated with cancer development [9, 10], some papers have suggested that genomic damage could trigger their upregulation [11–14], and overexpression of these proteins has been linked to higher tumor aggressiveness [12–14]. Wilczak et al. [14] showed that MMR gene overexpression is associated to poor outcome, and this relationship was more prevalent in neoplasms lacking the *TMPRSS2-ERG* fusion.

ERG overexpression resulting from the genetic rearrangement between *TMPRSS2* and *ERG* genes has been defined as the most frequent alteration in PrCa [17–19]. The relationship of *PTEN* loss with high-grade prostate neoplasms is widely reported [20, 21], both *ERG* rearrangements and loss of *PTEN* being concomitant steps in PrCa which can cooperate in progression [22–26]. *PTEN* inactivation has been considered a late event in PrCa carcinogenesis [27, 28].

There are few studies dealing with MMR defects outside the spectrum of Lynch Syndrome [5, 9–15]. This research field has become of particular relevance with the recent approval of an immunotherapy-based PD-1 inhibitor cancer treatment (Pembrolizumab) by the U.S. Food and Drug Administration (FDA) for patients with metastatic or unresectable solid tumors with MMR deficiency or MSI regardless of the histology ("tumor agnostic") [29–31].

Herein, the aims of the present study have been to analyze by means of immunohistochemistry (IHC) the expression of MSH2, MSH6, MLH1, and PMS2 and to investigate its association with ERG and PTEN expression in the same PrCa samples, with the goal of assessing the impact of these alterations on the clinical course of the disease.

Materials and methods

Patients and tumor samples

Two hundred and twenty-eight prostate tumors obtained from wholly mapped, formalin-fixed, paraffin-embedded (FFPE) radical prostatectomy specimens were retrospectively collected from the files of the Parc de Salut MAR Biobank (MARBiobanc, Barcelona, Spain). Tissue sections were retrieved from dominant tumor foci. According to the grade group (GG) PrCa grading system accepted by the WHO in 2016 [32], tumor samples were classified as GG1 ($n = 39$), GG2 ($n = 79$), GG3 ($n = 29$), GG4 ($n = 33$), and GG5 ($n = 20$).

TMA construction

A total of nine tissue microarrays (TMAs) were constructed using a manual tissue arrayer (Chemicon ATA-100), as previously described [33]. For each case, from 1 to 3 cores of the different Gleason score (GS) tumor areas were selected from the hematoxylin-eosin (H&E)-stained sections of donor blocks. From the nine resulting TMAs, 3-μm-thick sections were cut and transferred to glass slides, and H&E staining of the TMA sections was performed and reviewed by at least two expert pathologists (NJ, RA-G, ALl, and JLl) for diagnostic and grading confirmation.

Immunohistochemistry of mismatch repair proteins (MSH2, MSH6, MLH1, and PMS2), ERG, and PTEN

Immunohistochemical expression of the six proteins was assessed in 4.032 core sections (672 per antibody) derived from the 228 cases. In total, there were 28 non-informative cases. There was more than one evaluable core in 161 cases. From these 200 cases, there were only 5 cases which were not assessable for the MSH6 protein. Association between markers was assessed in individual cores.

Immunohistochemical analysis was carried out using anti-MSH2 mouse monoclonal antibody (clone G219-1129, Ventana, Roche, Tucson, AZ, USA), anti-MSH6 mouse monoclonal primary antibody (clone 44, Ventana, Roche, Tucson, AZ, USA), anti-MLH1 mouse monoclonal primary antibody (clone M1, Ventana, Roche, Tucson, AZ, USA), anti-PMS2 rabbit monoclonal antibody (clone EPR3947, Ventana, Roche, Tucson, AZ, USA), anti-ERG primary rabbit monoclonal antibody (clone EPR3864, Epitomics, Burlingame, CA, USA), and anti-PTEN mouse monoclonal antibody (clone 6H2.1, Dako) with the Dako Envision+ System-HRP (Dako, Glostrup, Denmark).

Immunostaining of MMR proteins was assessed using the histoscore method ($+1 \times$ (% cells 1+) $+ 2 \times$ (% cells 2+) $+ 3 \times$ (% cells 3+)), by which a continuous variable is obtained ranging from 0 to 300. We grouped the cases into two categories: group 0 (histoscore = 0–10) and group 1 (histoscore = > 10–300). Only nuclear staining was analyzed, and cytoplasmic reactivity was not considered [34]. We used lymphocytes and stromal and endothelial cells as internal positive controls. Finally, we selected a core per case using the highest GS and the lowest nuclear MMR histoscore as main criteria.

Regarding ERG immunostaining, only two patterns of nuclear expression were considered: negative (no detectable staining) or positive (detectable staining). We used endothelial cells as positive internal control. For PTEN immunostaining, a semi-quantitative scoring system was implemented: total expression loss = 0, partial expression loss = 1, and intense, homogeneous expression = 2. Adjacent normal tissue was used as an internal reference for intensity scoring.

Statistical analysis

Categorical variables are presented as frequencies and percentages, and quantitative variables as median and range. Pearson chi-square test or Fisher's exact test was used to assess the relationship between two categorical variables.

For the DNA MMR statistical analysis, expression of each of the four proteins was assessed either alone or evaluating only the dominant protein status (MHS2 and MLH1) in each heterodimer. Regarding the statistical analysis of ERG and PTEN, expression of each protein was assessed individually.

The relationship with PSA progression-free survival was analyzed using the Kaplan-Meier (log-rank) test in 200 patients. Patients were censored when an increase in serum PSA > 0.2 ng/ml was detected at the time of their last clinical follow-up appointment. The mean follow-up was 102.5 months (1–212 months). A p value < 0.05 was considered to be statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Immunohistochemical analysis of MMR proteins, ERG, and PTEN

In all the 200 assessable cases, the core with the highest grade was equivalent to the global tumor GG and this was the core used for all statistical comparisons. Normal prostate epithelium showed variable levels of nuclear MMR protein staining in both luminal and basal cells, particularly in the latter ones, and its intensity ranged from negative to moderate, being usually weak to negative. Representative images are shown in Fig. 1.

MMR protein expression was classified as negative for cases with histoscore (0 to 10) and positive for histoscore (> 10–300). Loss of nuclear expression was found in 8% (16/200) of the cases for MSH2, 5% (10/200) for MLH1, and only 2% (4/200) for PMS2. Three of 10 (30%) MLH1-deficient cases had also loss of PMS2 expression.

With regard to MSH6, a large proportion of cases did not show immunostaining for this molecule, both in the tumor and in the normal internal control cells. In addition, a subset of cases had an increase in MSH6 expression in the neoplastic cells as compared with the adjacent normal tissue control. Given the fact that the MSH6 staining pattern found in our PrCa series was different from that of the other proteins, we applied a different terminology: negative cases were classified as having "MSH6 basal levels" and the positive cases, i.e., those having a histoscore value higher than 10, were referred to as "overexpressing phenotype," a finding that was present in 42.1% (82/195) of cases.

ERG overexpression was detected in 48% (96/200) of the cases in our series, while PTEN expression loss was found in 36.5% (73/200).

Relationship between MMR, ERG, and PTEN protein expression and grade groups

Distribution of alterations in immunohistochemical expression of MMR, ERG, and PTEN proteins according to the GG classification is shown in Table 1. Loss of MSH2, MLH1, and PMS2 among the GG categories was rather homogeneous, with no statistical differences among them.

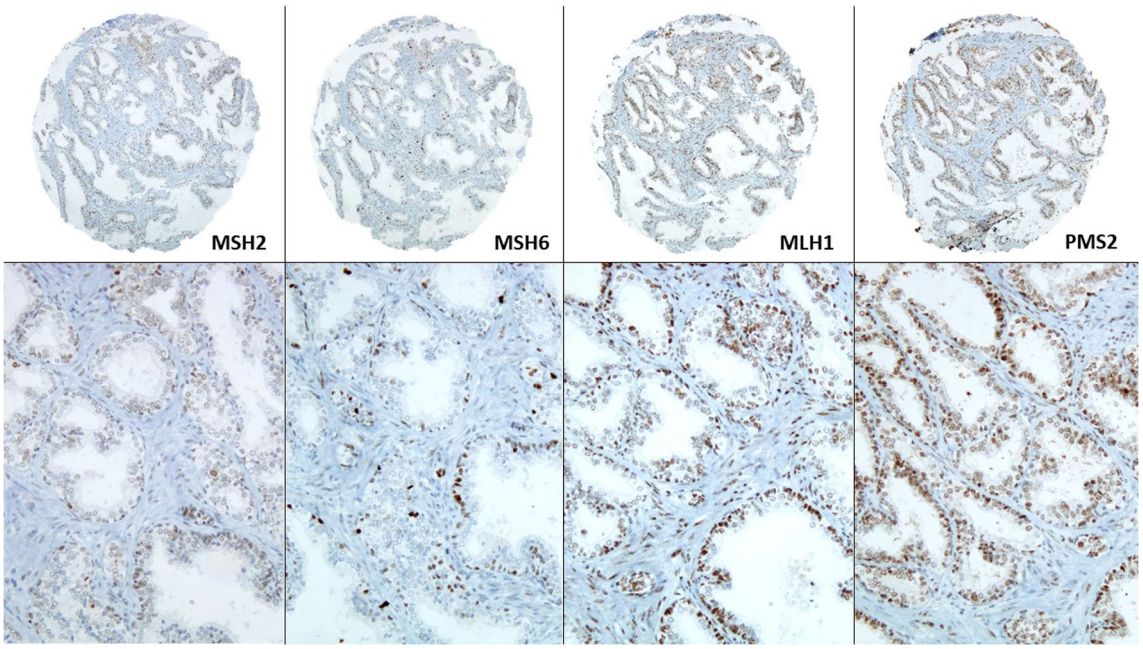

Fig. 1 Representative pictures of MSH2, MSH6, MLH1, and PMS2 immunostaining in normal prostatic epithelium (× 40, top row and × 150, bottom row)

Table 1 Prevalence of alterations in the mismatch repair, ERG and PTEN protein expression in prostate cancer according to grade group (GG) classification

Type of alteration	GG1 tumors	GG2 tumors	GG3 tumors	GG4 tumors	GG5 tumors	*p* value
MSH2 loss	2 (5.1%)	8 (10.1%)	3 (10.3%)	2 (6.1%)	1 (5%)	Fisher's exact test, $p = 0.877$
MSH6 overexpression	15 (41.7%)	30 (39%)	12 (41.4%)	12 (36.4%)	13 (65%)	Pearson chi-square, $p = 0.280$
MLH1 loss	3 (7.7%)	4 (5.1%)	2 (6.9%)	1 (3%)	0 (0%)	Fisher's exact test, $p = 0.780$
PMS2 loss	0 (0%)	3 (3.80%)	0 (0%)	1 (3%)	0 (0%)	Fisher's exact test, $p = 0.746$
ERG overexpression	17 (43.6%)	36 (45.6%)	16 (55.2%)	16 (48.5%)	11 (55%)	Pearson chi-square, $p = 0.776$
PTEN loss	8 (20.5%)	29 (36.7%)	11 (37.9%)	13 (39.4%)	12 (60%)	Pearson chi-square, $p = 0.056$

DNA MMR DNA mismatch repair, *GG* grade groups, *WHO* World Health Organization

Examples of cases with MMR immunostaining alterations are shown in Fig. 2.

As the percentages of MSH6 protein overexpression were very similar in GG1 through 4, we clustered them together and

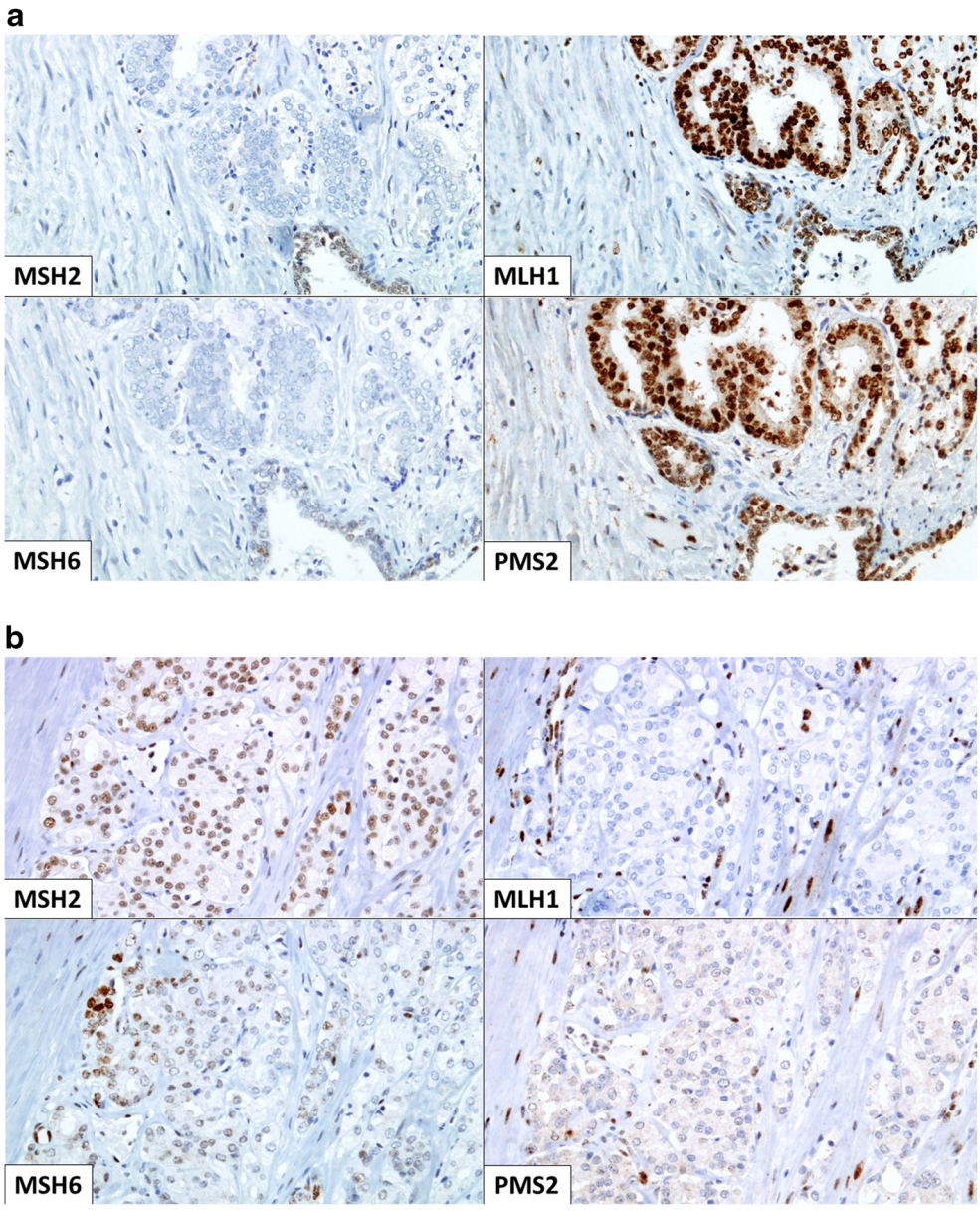

Fig. 2 MSH2, MSH6, MLH1, and PMS2 immunostaining in prostate cancer (× 200). **a** Case 1: MSH2 expression loss and MSH6 basal levels immunostaining, MLH1 and PMS2 fully preserved expression in a grade group 4 (GG4) tumor (Gleason score (GS) = 4 + 4). **b** Case 2: MLH1 and PMS2 expression loss, MSH2 expression and MSH6 overexpression in a GG4 tumor (GS = 4 + 4). Lymphocytes and stromal cells used as internal control

Table 2 Relationship between single and combined alterations in MSH2, MSH6, and MLH1 expression and ERG status

Type of alteration	Total of tumors	ERG-positive tumors	ERG-negative tumors (*wt*)	*p* value
MSH2 loss	16	3 (18.8%)	13 (81.2%)	Pearson chi-square, $p = 0.015$
MSH6 overexpression	82	45 (54.9%)	37 (45.1%)	Pearson chi-square, $p = 0.112$
MLH1 loss	10	3 (30%)	7 (70%)	Fisher's exact test, $p = 0.335$
MSH2 and/or MLH1 loss	21	5 (23.8%)	16 (76.2%)	Pearson chi-square, $p = 0.019$

compared with GG5. Overall, MSH6 overexpression was found in 39.4% (69/175) of the tumors in GG1–4 and in 65% (13/20) of the cases in GG5. This difference was statistically significant (Pearson chi-square, $p = 0.0281$).

ERG overexpression was detected in 43.6% (17/39) of GG1, 48.2% (68/141) of clustered GG2 to 4, and 55% (11/20) of GG5 (chi-square test, $p = 0.705$). The percentage of cases with PTEN expression loss was 20.5% (8/39) in GG1, 37.6% (53/141) of clustered GG2 to 4, and 60% (12/20) of GG5 (chi-square test, $p = 0.01$). Thus, ERG overexpression was not related to GG tumor classification, while PTEN expression loss was statistically more frequent in the higher-grade tumors.

Relationship between combinations of the MMR dominant proteins (MSH2, MLH1) and grade groups

Distribution of protein expression and loss was again rather homogeneous. Loss of MSH2 or MLH1 was observed in 2.6% of GG1, 10.1% of GG2, 10.3% of GG3, 9.1% of GG4, and 5% of GG5. Double loss of MSH2 plus MLH1 was a very uncommon event, only present in 5.1% of GG1, 2.5% of GG2, 3.4% of GG3, and completely absent in GG4 and GG5. Loss of expression of MSH2 or MLH1 alone or combined was not associated with any GG category ($p = 0.787$).

Relationship between MSH2, MSH6, and MLH1 protein expression and ERG status in prostate tumors (Table 2)

There were 96 ERG-positive and 104 ERG-negative (*wild-type*) (*wt*) cases. PMS2 was not included in the analysis because its loss was extremely uncommon.

With regard to MSH2, loss of expression was strongly associated with ERG *wt* cases ($p = 0.015$), with 81.2% of MSH2-negative cases being ERG *wt*.

Expression of MSH6 was not statistically related to ERG status ($p = 0.112$). Loss of MLH1 expression was much more frequent in ERG *wt* tumors (70%); however, this difference was not statistically significant ($p = 0.335$). Cases with MSH2 and/or MLH1 expression loss were significantly more common in the ERG *wt* group ($p = 0.019$).

Relationship between MSH2, MSH6, and MLH1 protein expression and PTEN status in prostate tumors

The relationship between MSH2, MSH6, and MLH1 expression and loss of PTEN expression was also analyzed. Loss of PMS2 expression was a very infrequent event, and we excluded it from the analysis. PTEN expression (PTEN *wt*) was present in 127 cases, and it was either completely negative or partially lost in 73. Data are summarized in Table 3.

MSH2, MSH6, MLH1, and PMS2 expression and PSA recurrence analysis in PrCa

A Kaplan-Meier analysis for PSA recurrence was performed for GG classification (log rank test, $p = 0.136$), MSH2 loss vs MSH2 *wt* (log rank test, $p = 0.564$), MSH6 overexpression vs MSH6 *wt* (log rank test, $p = 0.367$), MLH1 loss vs MLH1 *wt* (log rank test, $p = 0.548$), MSH2 and/or MLH1 loss vs MSH2 and MLH1 *wt* (log rank test, $p = 0.656$), double MSH2 plus MLH1 loss vs non-double loss (log rank test, $p = 0.335$), and PMS2 loss vs PMS2 *wt* (log rank test, $p = 0.011$). Despite the fact that the loss of PMS2 expression was a very infrequent event, there was a statistical association of this finding with a shorter PSA recurrence.

Table 3 Relationship between single and combined alterations in MSH2, MSH6, and MLH1 expression and PTEN status

Type of alteration	Total of tumors	PTEN-positive tumors (*wt*)	PTEN-negative tumors	*p* value
MSH2 loss	16	8 (50%)	8 (50%)	Pearson chi-square, $p = 0.242$
MSH6 overexpression	82	53 (64.6%)	29 (35.4%)	Pearson chi-square, $p = 0.896$
MLH1 loss	10	7 (70%)	3 (30%)	Pearson chi-square, $p = 0.749$
MSH2 and/or MLH1 loss	21	12 (57.1%)	9 (42.9%)	Pearson chi-square, $p = 0.522$

As MSH2 loss and the combination MSH2 and/or MLH1 loss were associated with ERG *wt* protein expression, we also analyzed the combinations: MSH2 loss/ERG *wt* vs ERG+ (MSH2 loss or *wt*) vs MSH2/ERG *wt* (log rank test, $p = 0.679$), and MSH2 and/or MLH1 loss/ERG *wt* vs ERG+ (MSH2 and/or MLH1 loss or *wt*) vs MSH2/MLH1/ERG *wt* (log rank test, $p = 0.797$).

As MSH6 overexpression was statistically associated with GG5 tumors, a Kaplan-Meier analysis for PSA recurrence comparing MSH6 overexpression vs MSH6 *wt* was also performed (log rank test, $p = 0.1$). Despite the fact that there was no statistical significance, MSH6 overexpression seems to be a marker of worse prognosis in GG5 tumors (Fig. 3).

Discussion

The role of MMR genes and their respective proteins has been extensively addressed in colorectal and endometrial cancer [6, 7]. For both cancers, a considerable group of hypermutated tumors was associated with MSI, most frequently due to *MLH1* epigenetic silencing. However, there is controversy about the role of MMR genes in the development and progression of PrCa, as genetic alterations leading to MSI are less well defined in this type of cancer. There are few reports of defects in MMR protein expression in PrCa [9–15], and the underlying mechanisms conditioning these deficiencies and their clinical-pathological impact deserve further investigation.

In this study, we have analyzed the immunohistochemical expression of MSH2, MSH6, MLH1, and PMS2 in a large series of prostate tumors in order to determine the distribution of single and combined alterations of these proteins among the GG and their association with ERG expression and PTEN loss. To the best of our knowledge, our study is the first to analyze the MMR protein expression according to the GG, the new PrCa grading system adopted by the WHO 2016 [32]. In addition, it is one of the first to analyze the relationship between MMR protein expression in PrCa and ERG and PTEN status.

In keeping with previous studies [9, 12, 14], a low frequency of MSH2, MLH1, and PMS2 protein expression loss was found in our PrCa subset. Loss of protein expression was detected in 8% for MSH2, 5% for MLH1, and 2% for PMS2, with no statistical differences among the GG categories. For MSH6, due to the peculiar pattern of expression in our series of PrCa, we hypothesized that negative immunostaining could be considered as basal levels in this setting and therefore the detection of a positive immunostaining could be classified as overexpression. A high percentage (42.1%) of tumors showed MSH6 overexpression. As percentages of MSH6 overexpression were very similar in GG from 1 through 4, we also carried out an analysis comparing pooled GG1–4 with GG5. With this approach, a statistical association between MSH6 overexpression and GG5 tumors was found (Pearson chi-square, $p = 0.0281$). Thus, MSH6 overexpression was significantly higher in the cases with a more aggressive phenotype and probably with a higher genomic damage.

Recently, MSH2 protein loss [9] has been reported in primary prostate tumors, more commonly in high-grade tumors. Basu et al. [35] reported a significantly lower expression of *MSH2*, *MSH6*, and *MLH1* genes in prostate tumors, proposing that this deficiency would be a hallmark distinguishing PrCa from benign prostatic hyperplasia.

Interestingly, while some studies reported an association between loss of function of MMR genes and less favorable PrCa

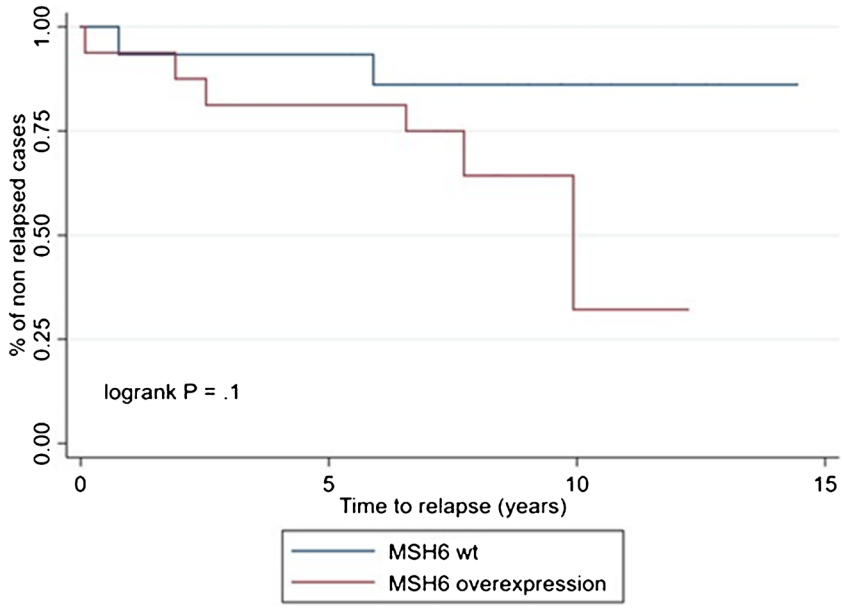

Fig. 3 A Kaplan-Meier analysis for PSA recurrence analysis comparing MSH6 overexpression vs MSH6 *wild type* was performed in the GG5 tumor group (log rank test, $p = 0.1$)

features [9, 16], other authors hypothesized that genomic damage could trigger MMR gene upregulation, linking overexpression to higher tumor aggressiveness and poor outcome [12–14]. Overexpression of MSH2 and/or MLH1 has been previously reported in other tumors [36–38]. In PrCa, Velasco et al. [39] reported higher staining intensity of MSH2 in prostate tumor samples compared to normal glands and benign prostatic hyperplasia. Norris et al. [11] also documented that increased PMS2 was a prognostic marker in pre-neoplastic and PrCa tissue. In this regard, we found MSH6 overexpression in about 42% of prostate tumors, with an association with the more aggressive cases. We speculate that MSH6 protein would be overexpressed in this setting because the increased DNA replication disarrangements require an efficient DNA repair system, a hypothesis that would be in accordance with previous studies [11–15]. Although the molecular mechanisms controlling variable expression of MSH6 among the different GG are still not well known and require additional studies, our results seem to suggest that MSH6 could be a biomarker of genomic damage and aggressiveness in PrCa. In any event, it is really intriguing that on one hand we had cases in which both the tumor and the normal tissue lack MSH6 expression, and on the other hand, we had cases with weak expression in the normal tissue and obvious overexpression in the tumor tissue. This difference between normal and tumor would indicate that this is a real overexpression and it could be speculated, as it has been done in some papers [12–14], that this feature is a reflection of higher tumor aggressiveness, perhaps related to an accumulation of genetic events.

Genetic translocations involving promoters of androgen regulated genes and ETS family have been defined as the most common alteration in PrCa. Fusion between *TMPRSS2* and *ERG* is the most frequent one, accounting approximately for 90% of fusion-associated prostate tumors [17, 19, 40–42]. Likewise, it has been also reported that *ERG* fusions and loss of *PTEN* are common and concomitant alterations which can cooperate in PrCa development [22–26, 43], *PTEN* loss being a subclonal event after *ERG* gene fusion in a well-established prostatic tumor clone [44].

Recently, Wilczak and colleagues [14] showed that high expression levels of MSH6, MLH1, and PMS2 were frequent in PrCa and particularly strong in cancers with advanced pathological grade and stage, and early biochemical recurrence. This association was much stronger in cancers lacking *TMPRSS2-ERG* fusion ($p < 0.0001$), while MMR gene overexpression was only marginal in ERG-positive cancers.

In the present report, with the aim of assessing the impact of MMR protein alterations on the ERG pathway tumors, we have analyzed the relationship of MSH2, MSH6, MLH1, and PMS2 expression alterations with ERG and PTEN status. Our results revealed a statistical association between loss of MSH2 and ERG *wt*. Moreover, double loss of MSH2/MLH1, or the single loss of one of them, was also statistically associated with ERG *wt* status. Regarding MSH6, its overexpression was not associated with ERG or PTEN status. These results suggest a potential role for MMR protein alterations in an alternative PrCa pathway, since prostate tumors with MSH2 expression loss or combined deficiency of MSH2 and/or MLH1 could evolve through a carcinogenic PrCa pathway independent of ERG. In contrast, in our study, PTEN loss was not statistically related with the MMR protein expression. In this regard, PTEN loss is an important step in the pathogenesis and progression of PrCa, but it does not define by itself a pathway in the carcinogenesis of this tumor.

Finally, we investigated the potential relationship of our findings with PSA recurrence. Only loss of PMS2 expression, despite being an infrequent event, showed a statistical association with a shorter PSA progression-free survival.

In the current setting of personalized medicine, a better understanding of MMR deficiency leading to MSI is becoming more important with the recent approval of an immunotherapy-based PD-1 inhibitor molecule by the FDA [29], which could be beneficial in tumors with these alterations [30].

In conclusion, the results of the present study suggest the existence of an alternative non-*ERG* pathway associated with loss of MSH2 or MLH1 expression. On the other hand, MSH6 overexpression could be a marker for genomic damage and aggressiveness in PrCa. However, further studies are needed to determine its role in prostatic carcinogenesis and its possible applicability in the clinical practice. The immunohistochemical assessment of DNA MMR protein expression together with ERG and PTEN status could identify PrCa subgroups with different altered pathways, a fact that could contribute to improve patient stratification.

Conflict of interest The authors declare that they have no conflict of interest.

Authors' contributions RA-G, NJ, ML, ALl, LF, LC, and JLl-T contributed to specimen preparation and clinical data acquisition. JLl-T and SH-Ll designed the research study. RA-G, NJ, ALl, LS, and JLl-T participated in the pathological data management and staining evaluation. ML constructed and stained the tissue microarrays. XD and SH-Ll performed the statistical analysis. RA-G, SH-Ll, and JLl-T participated in the data preparation and analysis and wrote the paper. All authors have read and approved the final version of the manuscript.

Funding The study has been funded with grants Jordi Gras 2016-2017 from the Institut Hospital del Mar d'Investigacions Mèdiques (IMIM) and FIS/Instituto Carlos III/PI15/FEDER PI15/00452 from the Spanish Ministry of Health. The authors thank the Parc de Salut MAR Biobank (MARBiobanc), Barcelona, Spain, for the archival support.

Compliance with ethical standards

National and international guidelines (code of ethics, Declaration of Helsinki) and legal regulations on data confidentiality (Spanish Organic Law 15/1999 of December 13 on Protection of Personal Data [LOPD]) have been followed.

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