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DNA mismatch repair in cancer☆

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ABSTRACT

Microsatellite instability (MSI) refers to the hypermutator phenotype secondary to frequent polymorphism in short repetitive DNA sequences and single nucleotide substitution, as consequence of DNA mismatch repair (MMR) deficiency. MSI secondary to germline mutation in DNA MMR proteins is the molecular fingerprint of Lynch syndrome (LS), while epigenetic inactivation of these genes is more commonly found in sporadic MSI tumors. MSI occurs at different frequencies across malignancies, although original methods to assess MSI or MMR deficiency have been developed mostly in LS related cancers. Here we will discuss the current methods to detect MSI/MMR deficiency with a focus of new tools which are emerging as highly sensitive detector for MSI across multiple tumor types.

Due to high frequencies of non-synonymous mutations, the presence of frameshift-mutated neoantigens, which can trigger a more robust and long-lasting immune response and strong TIL infiltration with tumor eradication, MSI has emerged as an important predictor of sensitivity for immunotherapy-based strategies, as showed by the recent FDA's first histology agnostic-accelerated approval to immune checkpoint inhibitors for refractory, adult and pediatric, MMR deficient (dMMR) or MSI high (MSI-H) tumors. Moreover, it is known that MSI status may predict cancer response/resistance to certain chemotherapies.

Here we will describe the complex interplay between the genetic and clinical-pathological features of MSI/dMMR tumors and the cancer immunotherapy, with a focus on the predictive and prognostic role of MMR status for immune checkpoint inhibitors (ICIs) and providing some suggestions on how to conceive better predictive markers for immunotherapy in the next future.

Contents

Abbreviations: 5-FU, 5-fluorouracil; bMMRD, Biallelic mismatch repair deficiency; CD28, cluster of differentiation 28; CIN, Chromosomal instable; CR, complete response; CRC, colorectal cancer; CTL, cytotoxic T-lymphocytes; CTLA-4, cytotoxic T lymphocyte antigen-4; DFS, disease free survival; dMMR, deficient MMR; EC, endometrial cancer; EPCAM, epithelial cell adhesion molecule; FDA, Food and Drug Administration; GB, glioblastoma; GC, gastric cancer; HNPCC, hereditary nonpolyposis colorectal cancer; HR, Hazard ratio; ICI, immune checkpoint inhibitor; IHC, immunohistochemistry; IPI, ipilimumab; LS, Lynch syndrome; MANA, mutation-associated neoantigens; Mb, megabase; mCRC, metastatic colorectal cancer; MHC, major histocompatibility complex; MMR, mismatch repair; MSH2, MutS protein homologue 2; MSH6, MutS homologue 6; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, MSI-low; MSS, microsatellite stable; NCCN, National Comprehensive Cancer Network; NCI, National Cancer Institute; NGS, Next-generation sequencing; Nivo, nivolumab; NSCLC, non-small cell lung cancer; ORR, overall response rate; OS, overall survival; PCR, polymerase chain reaction; PD-1, programmed cell death 1; PD-L1, Programmed cell death ligand 1; PFS, progression free survival; pMMR, Proficient MMR; PMS2, PMS1 homologue 2; PR, partial response; TCGA, The Cancer Genome Atlas; TILs, tumor-infiltrating lymphocytes; TMB, tumor mutation burden; TME, tumor microenvironment; Tregs, regulatory T cells; WES, whole-exome sequencing.

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1. Introduction

1.1. The mismatch repair system

Microsatellites are short, tandemly repeated (usually 10–60 times) sequences of mononucleotide, dinucleotide or higher order nucleotide repeats (unit length ranging from 1 to 6 bases), that are scattered throughout the human genome, most commonly as (CA)n. (Findeisen et al., 2005;). These sites are prone to DNA replication errors as a result of DNA polymerase slippage, byYamamoto & Imai, 2015 either inserting additional bases when slippage occurs on the synthesized strand or by removing bases when slippage occurs on the template strand, leading to mismatched DNA strands (Drake, Charlesworth, Charlesworth, & Crow, 1998; Kunkel & Erie, 2005). It is estimated that the replicative DNA polymerases *epsilon* and *delta* make errors approximately once for every 10^4 and 10^5 nucleotides that they polymerize (Chang, Metzgar, Wills, & Boland, 2001). Thus, each time a cell divides, approximately 100,000 polymerase errors occur, which must be corrected through the combined actions of proofreading activity of polymerase *epsilon* and *delta* (Bebenek & Kunkel, 2004). However, some errors always escape proofreading, and are effectively corrected through the mismatch repair (MMR) system (Arana & Kunkel, 2010), responsible for the surveillance and correction of errors during DNA replication, repair and recombination.

MLH1, MutS protein homologue 2 (MSH2), MutS homologue 6 (MSH6) and PMS1 homologue 2 (PMS2) are the main proteins involved in this MMR system, and they interact as heterodimers: MSH2 couples with either MSH6 or MSH3 (forming MutSα and MutSβ complexes, respectively), and MLH1 couples with PMS2 or MLH3 (forming MutLα, MutLβ or MutLγ complexes, respectively) (Jiricny, 2006a, b; Li, 2008). The complex formed by a MutS and a MutL is ultimately responsible for the recognition of mismatches and insertion–deletion loops (Genschel, Littman, Drummond, & Modrich, 1998) and subsequent recruitment of the MLH1/PMS2 complex will degrade the mutated stretch and initiates resynthesis.

Patients with a defect in any of these components or, in a gene upstream of MSH2 that encodes the epithelial cell adhesion molecule (EPCAM), will develop a “mutator phenotype” with numerous frameshift mutations in coding and non-coding microsatellites and at other genetic loci beyond the microsatellites. This results in the microsatellite instability-high (MSI-H) phenotype, closely related to carcinogenicity of hereditary and sporadic tumors (Eshleman & Markowitz, 1996). MSI-H cancers are associated with 100- to 1000-fold increased mutation rates of frameshift and missense mutations.

Frameshift mutations in coding sequences may give rise to altered protein products in the tumor, termed “neoantigens”. These neoantigens are unique to the tumor and thus could potentially be recognized as “non-self” molecules by the immune system.

The purpose of this review is to examine current knowledge of the DNA MMR machine, review the landscape of tumors with MSI and outline the assays available to detect MSI or MMR deficiency. It is now widely recognized that the development of immune checkpoint inhibitors (ICIs) has revolutionized cancer care, showing unprecedented clinical benefit for some tumor types, including MSI-H cancers. In this review we will describe the relationship between MMR status and sensitivity to current immunotherapies, and we will address the immune contexture and genomic profiles of tumors with MSI and their implications for immunotherapeutic approaches.

2. Etiology of MSI-H tumors

2.1. Lynch syndrome-related and sporadic MSI-H tumors

MSI can be used as a surrogate marker of Lynch syndrome (LS) (also known as hereditary nonpolyposis colorectal cancer-HNPCC) since LS is caused by germline mutations in any one of five DNA MMR genes—MLH1, MSH2, MSH6 and PMS2 and, rarely, PMS1 (Fishel et al., 1993; Ionov, Peinado, Malkhosyan, Shibata, & Perucho, 1993; Kolodner et al., 1999; Leach et al., 1993; Liu et al., 1996; Papadopoulos et al., 1994).

In addition, germline deletions affecting the 3′ exon of EPCAM gene (previously known as TACSTD1, tumor-associated calcium signal transducer 1), result in transcriptional read-through and induce epigenetic silencing of the downstream MSH2 locus by promoter hypermethylation (Ligtenberg et al., 2009). Recently, germline mutations in MSH3 were reported, which occurs in longer repeats not routinely investigated by conventional MSI testing, further expanding the spectrum of MMR deficiency in humans (Adam et al., 2016). If, according to Knudson's second hit model, the remaining wild-type allele is somatically inactivated in LS patients, the DNA MMR capacity is lost (Knudson Jr., 1985).

Lynch syndrome is an autosomal dominant disorder characterized by an elevated risk for cancers of the ovaries, kidneys, bladder, stomach, small bowel, bile ducts, brain kidney, biliary tract and gallbladder cancers, and skin sebaceous tumors., with the biggest increase in risk for endometrial cancer (EC) and colorectal cancer (CRC) (Aaltonen et al., 1998; Lin et al., 1998; Salovaara et al., 2000).

In the mid-1960s Henry T. Lynch was the first to clinically describe this hereditary cancer syndrome and differentiated it from familial adenomatous polyposis (caused by an inherited mutation of the tumor suppressor gene APC) (Lynch, 1999; Lynch & de la Chapelle, 1999; Lynch & Smyrk, 1999).

Germline mutations in MMR genes lead to a cumulative risk of 60%–70% to develop CRC in men, and 30–40% in women; 40–80% is the cumulative risk of developing EC (Stoffel et al., 2009). It is, indeed, clinically important to emphasize that the lifetime risk of developing EC in affected women is higher than their lifetime risk of developing CRC (Hampel et al., 2005).

While LS has an incidence of 1:1000 in the general population, the incidence is up to 1:100 in individuals with CRC, accounting for about 2.5% of CRC (Chen et al., 2006; Ligtenberg et al., 2009).

Since about 15% of all CRC are MSI-H, the remaining 12.5% result from sporadic alteration in MMR genes (Koopman et al., 2009). The prevalence of MSI in CRC, however, differs based on tumor stage: about 20% in stage II, 12% in stage III and much less common among patients in stage IV (about 4%).

Sporadic MSI most commonly arises from epigenetic silencing of the MLH1 promoter, by aberrant methylation in CpG Island and is associated with a somatic BRAF pV600E mutation (Herman et al., 1998). Less commonly, sporadic cases are associated with biallelic somatic inactivation of the genes encoding MMR components (Nicolaides et al., 1994).

In regard to EC, MMR function is lost in 20–30% of patients (Chen et al., 2006; Palomaki, McClain, Melillo, Hampel, & Thibodeau, 2009), and LS accounts for approximately 25% of these cases, while the majority involve somatic hypermethylation of MLH1 promoter or somatic mutations of MMR genes (Koopman et al., 2009; Kuismanen et al., 2002; Kunitomi et al., 2017). The incidence of MSI in gastric cancers (GC)

varies from 8 to 37% and the MSI phenotype in GC is predominantly caused by epigenetic hypermethylation of MLH1 rather than germline mutations in MMR genes (Seo et al., 2009).

There is an interesting relationship between genotype and phenotype, as peculiar manifestation of LS reflects the specific mutation carried by the patient. Patients with MSH2 mutation, for instance, have a greater risk of developing non-CRC (Ligtenberg et al., 2009) compared to patients with MLH1 mutation, which are more likely to develop CRC at younger age (Lin et al., 1998). Finally, EC risk is higher for patients with MSH6 germline mutations (Kolodner et al., 1999). Comparison of instability frequencies of the individual marker loci between CRC and EC showed that despite identical genetic predisposition, the MSI profiles of these tumors show significant differences. CRCs present a predominant instability at the non-coding BAT loci (at least one being unstable in 89% of tumors), TGFRII (73%), dinucleotide repeats (at least one being unstable in 70% of tumors), MSH3 (40%), and BAX (30%). On the other hand, PTEN instability is significantly associated with ECs, occurring in 20% of these tumors, as compared to 5% of CRC (Kuismanen et al., 2002). In ECs, however, the pattern tends to be more heterogeneous, typically involving different coding repeats. This implies that the genesis of gastrointestinal and ECs occurs by different routes even if driven by generalized MSI.

3. The methods of testing MSI and MMR deficiency and sensitivity properties

3.1. Clinical criteria for the diagnosis of Lynch syndrome

Amsterdam and Bethesda criteria were developed to identify potential LS patient candidates for genetic testing. The Amsterdam criteria, first set in 1991, were developed to clinically identify potential HNPCC patients (Vasen, Mecklin, Khan, & Lynch, 1991; Vasen & Muller, 1991).

Advances in knowledge of HNPCC, make it clear that this syndrome was associated also with higher risk of extracolonic cancers. In order to address this issue, in 1998 the revised Amsterdam criteria (Amsterdam II) were developed, as following: diagnosis of CRC in two first degree relatives, involving at least two generations, one of these must have been diagnosed by the age of 55. The presence of a third member of the family with an unusual early-onset tumor or EC is considered sufficient to classify the family as meeting the Amsterdam II criteria (Aaltonen et al., 1993).

The Bethesda guidelines were proposed first in 1997 and further revised in 2003 (Boland et al., 1998; Umar, Boland, et al., 2004; Umar, Risinger, Hawk, & Barrett, 2004) to overcome the limitations of the Amsterdam criteria which did capture all patients with possible LS. Only one of the following criteria need to be met in the revised Bethesda guidelines: diagnosis of CRC under the age of 50 years, presence of other LS–related tumors (including synchronous or metachronous CRC), patient under the age of 60 with CRC histology that resembles the typical characteristic of MSI tumor, diagnosis of CRC in a patient with history of first-degree relative under the age of 50 with a LS–related cancer, or a CRC patient with at least two first- or second-degree relatives with LS–related cancers. Of course, all patients who meet the Amsterdam criteria are also recommended to undergo further testing. It has been shown that the Bethesda criteria are more sensitive (94%) as compared to Amsterdam II criteria (72%) and the original Amsterdam criteria (61%) identifying patients with LS (Moreira et al., 2012). However, the specificity of these criteria is low and about 50% of the clinically suspected cases are not confirmed by a genetic defect (Menko, Wijnen, Vasen, Sijmons, & Khan, 1998; Wijnen et al., 1998). Dinh et al. considered the opposite approach of general population screening using a risk prediction tool (PREMM126) and reported that screening of individuals with a predicted risk of LS ≥ 5% was cost/effective, regardless of the age (Dinh et al., 2011).

Based on all these data, in 2018 National Comprehensive Cancer Network (NCCN) guidelines have been published. This guideline endorses universal MMR or MSI testing of all patients who have a personal history of CRC (National Comprehensive Cancer Network, 2018). Many NCCN member institutions even suggest to perform IHC and sometimes MSI testing on all newly diagnosed CRC and EC, regardless of family history, to determine which patients should further being evaluated for LS.

3.2. MSI testing

Screening to determine defective MMR status is becoming increasingly common, having important implications not only for screening for LS, but also for prognosis, and for prediction of response to fluorouracil and ICIs therapy.

MSI polymerase chain reaction (PCR) and immunohistochemistry (IHC) are two molecular biology–based methods that are in routine use for clinical MSI testing. MSI-PCR analysis is used to detect instability in microsatellite repeats whereas MMR IHC is used to detect the lack of expression of one or more MMR proteins (Aaltonen et al., 1998; Cairns et al., 2010).

MSI is detected by PCR amplification of specific microsatellite repeats, whose instability is determined by comparing the length of nucleotide repeats in tumor cells and normal cells from adjacent normal mucosa. These regions are amplified within both tumor and normal tissue via fluorescent multiplex PCR and their size assessed by capillary electrophoresis (Bacher et al., 2004; Berg et al., 2000).

In the mid-1990s, investigators were adopting different markers, thus resulting in a great variability of MSI frequency reported even in the same type of cancer. To overcome this problem, in 1997 the National Cancer Institute (NCI) has recommended a panel (known as NCI or Bethesda panel) of five microsatellite markers for testing: two mononucleotides loci (Big Adenine Tract [BAT] 25 and BAT26 and three dinucleotide repeats (D2S123, D5S346 and D17S250) (Umar, Boland, et al., 2004; Umar, Risinger, et al., 2004). Three categories of MSI have been established based on the results: MSI-high (MSI-H) indicating a shift in the size of at least two of the five microsatellite loci in tumor as compared to normal tissue (or >30% of loci if a larger panel of markers is used); microsatellite stable (MSS), indicating no loci with instability (or <10% of loci in larger panels) and MSI-low (MSI-L), associated to a shift in the size of one locus (or in 10–30% of loci in larger panels). MSI-L behaves in a similar manner to MSS tumors and does not appear to be a good predictor of LS, so this result is grouped with the MSS type and does not lead to further testing. It has been shown that the dinucleotide repeats have less sensitivity and specificity than mononucleotide repeats, in particular in patients with non CRC, and in MSH6 mutation-related tumors (Verma et al., 1999). The reason for this is that MSH6 protein is involved in the repair of base–base mismatches, but not dinucleotide ones, as well as in single nucleotide deletions/insertions, but not in the repair of larger deletion/insertions and consequently MSH6-deficient tumors can result falsely stable when analyzed using the three dinucleotide markers in the NCI panel (Verma et al., 1999).

A recent follow-up NCI workshop recognized this limitation of the original Bethesda panel (Umar, Risinger, et al., 2004). Indeed, a panel of five mononucleotide repeats (NR-21, NR-22, NR-24, BAT-25, BAT-26) was proposed (Suraweera et al., 2002). A modified pentaplex panel with replacement of NR-22 with NR-27 is also used. They showed that the pentaplex assay efficiently discriminates the MSI status of tumors regardless of their MMR defect and consequently, the pentaplex panel was developed as a procedure with higher sensitivity and specificity, and has been proposed as a replacement for the Bethesda panel (Buhard, Suraweera, Lectard, Duval, & Hamelin, 2004).

An alternative panel has been developed by the Promega Corporation, the MSI Analysis System, which is a fluorescent multiplex assay using five mononucleotide microsatellite markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) based on a study conducted by Bacher et al., comparing the sensitivity and specificity a set of 266 mono-, di-, tetra-, and penta-nucleotide microsatellite loci for MSI screening

(Bacher et al., 2004). This research determined that mononucleotide markers were more sensitive and specific than dinucleotide markers for the detection of MSI. A study conducted at Johns Hopkins independently confirm the high performance of this assay (Murphy et al., 2006).

Controversy still exist about the most appropriate panel for screening MSI, since a recent work reached opposite conclusions and suggest that a panel targeting longer loci may have improved sensitivity (Dudley, Lin, Le, & Eshleman, 2016). Moreover, regardless of the panel used and the length of loci, since MSI testing is based on specific microsatellites analysis, the test is hampered by a missing rate estimate around 0.3% to 10% of cases (Berg et al., 2000).

3.3. Immunohistochemistry

IHC analysis of MMR proteins (MLH1, MSH2, MSH6 and PMS2) is commonly used as alternative to MSI to detect MMR deficiency in clinical practice and can inform genetic testing for LS (Baudhuin, Burgart, Leontovich, & Thibodeau, 2005). IHC for MMR proteins has comparable performance characteristics to MSI testing and high concordance rate, with 100% specificity to MSI-H tumors and 96.7% for MSS and MSI-L tumors (Remo, Fassan, & Lanza, 2016; Wang et al., 2017). Lack of expression of one or more of MMR proteins leads to a diagnosis of deficient MMR (dMMR) while suggesting which encoding gene is most likely mutated or inactivated. In fact, MLH1 and MSH2 proteins are stable without their dimer partners (PMS2 and MSH6, respectively), but the reverse is generally not true. As a result, tumors with absent expression of MLH1 and PMS2, but retained expression of MSH2 and MSH6, represent deficient MLH1 expression, where the lack of expression of PMS2 is consequence of MLH1 deficit (either by promoter hypermethylation or mutation). On the other hand, if mutations occur in PMS2 or MSH6, only the affected protein will be lost (however, MSH2 may be lost when both of its binding partners, MSH6 and MSH3, are lost) (Vilar & Gruber, 2010).

These features allow one to determine by IHC which of the MMR genes is likely mutated (or methylated), an advantage not present with MSI testing (Shia, 2008; Zhang, 2008). MSI analysis can also be more time- and labor-consuming than IHC.

However, pros and cons are inherent to either strategy. In fact, about 5% to 11% of MSI cases will not show MMR protein loss, because missense mutations in the MMR gene can lead to functional inactivation of the protein without affecting its stability and antigenicity and, therefore, its expression level. Some analyses, evaluating cost spent per life-year gained in the general population, have favored PCR-based MSI testing as an initial measure (Mvundura, Grosse, Hampel, & Palomaki, 2010) whereas a more recent cost-effectiveness study favored screening patients with IHC (Ladabaum et al., 2011; Snowsill et al., 2015). These older analyses do not take into consideration cost-effectiveness in the context of an effective immunotherapy option for these patients.

The Association of Molecular Pathology supports the idea that IHC and MSI screening methods provide complementary information regarding defective MMR, and thus recommend all new CRC cases to be subjected to concurrent MSI analysis, IHC for MMR proteins, and BRAF mutation screening (as discussed in the next paragraphs), although whether this is the most cost-effective approach in the general population, is still debatable (Mills et al., 2014). Snowsill et al. clearly showed that all strategies included for the identification of LS (MSI testing, IHC, BRAF mutation) were cost-effective versus no testing.

3.4. The role of BRAF and/or MLH1 promoter hypermethylation in sporadic MSI-H CRC

The immunohistochemical finding in MSI-H sporadic CRC is usually simultaneous loss of MLH1 and PMS2. This is caused by MLH1 promoter hypermethylation, which is often associated with a BRAF mutational hotspot in nucleotide 1796 within exon 15, accounting for a T:A transversion mutation and a valine to glutamic acid substitution (c.1799T > A -p.V600E-), which is the most frequent somatic substitution identified in dMMR CRC (Davies et al., 2002; Yuen et al., 2002). Of note, BRAF mutations are mutually exclusive with KRAS mutations (Miyaki et al., 2004).

Promoter hypermethylation of hMLH1 and subsequent BRAF V600E alterations have been reported in about 10% to 15% of MMR proficient (pMMR) tumors, and in 70% of dMMR tumors (Wang et al., 2003).

Given that BRAF V600E mutation occurs at a higher frequency in sporadic MSI tumors than in hereditary cases, clinicians have used it to support the sporadic origin of MSI tumors (Domingo et al., 2004). However, although a BRAF mutation profoundly reduces the probability of a diagnosis of LS, it does not entirely exclude the possibility (Funkhouser Jr. et al., 2012).

3.5. Assessing MSI using next generation sequencing

Current available tools have been primarily developed and optimized for the detection of MSI in CRC (Bartley, Luthra, Saraiya, Urbauer, & Broaddus, 2012; de la Chapelle & Hampel, 2010; Haraldsdottir et al., 2014; Salipante, Scroggins, Hampel, Turner, & Pritchard, 2014), hence their ability to detect instability events in other cancer types is a matter of dispute. For instance, evidence exists that MSI testing by PCR may be less accurate in tumor types other than CRC (Faulkner, Seedhouse, Das-Gupta, & Russell, 2004). It is clear that there is a great variability in microsatellite loci instability among different cancer types, and loci that are consistently stable in CRC may be frequently mutated in other cancer types, and vice versa (Forgacs et al., 2001; Kim, Laird, & Park, 2013; Onda et al., 2001). This cancer-specific MSI landscape has important implications and challenges for the diagnosis of MSI in clinical practice.

Next-generation sequencing (NGS) with targeted gene sequencing or whole exome/genome sequencing have emerged as a new tool for identification of patients with DNA-MMR deficiency, by comparing sequencing reads around microsatellite regions in the tumor and the matched normal, or by counting mutations identified in exons, respectively (Salipante et al., 2014; Timmermann et al., 2010; Woerner et al., 2010). NGS, allowing to investigate a myriad of microsatellites simultaneously, is more comprehensive and it is not cancer-type-specific, potentially being a better strategy to ascertain instability burden and MSI status in all cancer types, and overcoming the sensitivity and specificity issues related to both PCR-based MSI testing and IHC testing for MMR proteins.

Moreover, NGS can be applied to formalin-fixed and paraffin embedded (FFPE) tissue material as well as highly degraded DNA which is routinely prepared in pathology departments or found in archived DNA (Stiller, Knapp, Stenzel, Hofreiter, & Meyer, 2009).

Different computational tools have been developed (Hause, Pritchard, Shendure, & Salipante, 2016; Hechtman et al., 2017; Huang et al., 2015; Kautto et al., 2017; Niu et al., 2014). Here we will discuss the most recent data obtained with these tools, providing a paradigm of how they can be profiled to serve as highly sensitive detector for MSI across multiple tumor types.

Hause et al. developed the MOSAIC method for cross-sectional MSI analysis in 18 cancer types using the cancer exomes from The Cancer Genome Atlas (TCGA) database, thus extending the analysis to additional cancer types for which MSI status is not tested routinely in clinical practice. In this study authors investigated a total of 223,082 microsatellites, from 5930 tumor exomes. The average number of unstable microsatellite loci varied considerably by cancer type (765 in thyroid carcinomas, to 2315 in colon cancer). As expected the highest prevalence of MSI-H cases occurred in cancer types that classically demonstrate MSI: EC (30%), GC (19%) and colon (19%), while rectal cancers had a lower prevalence of MSI-H specimens (3%). Low but still detectable, frequencies of MSI-H were observed in 12 other cancer types, suggesting that MSI may be a generalized cancer phenotype. They used a set of 617 specimens (colon, rectal, endometrial, stomach) as control

and compared the results obtained with MSI–PCR and MOSAIC, showing 95.8% sensitivity and 97.6% specificity (Hause et al., 2016).

As we will discuss later, the prognostic role of MSI status is still a matter of dispute, with most of the data available assessed in CRC patients (Samowitz et al., 2001). A very interesting finding from Hause et al. was that, while only a weak association was observed between MSI status and survival outcome, the global burden of microsatellites (measured as continuous variable), showed a more significant positive correlation with survival in uterine, endometrial, rectal, colon, stomach, and thyroid cancer and lower-grade glioma. This result may reflect a link between MSI events and the production of cancer neoantigens that can be recognized as 'non-self' by the immune system (Mlecnik et al., 2016).

Finally, the fractions of unstable loci were not significantly different between metastatic and primary tumors, consistent with other studies (Fujiyoshi et al., 2017; Haraldsdottir et al., 2016; Jung et al., 2017) which suggests that either the primary tumor or the metastasis can be assessed for MSI.

In 2017 Bonneville et al., expanded our knowledge investigating the prevalence of MSI in 21 additional cancer types by using the previously published MSI-calling tool, MANTIS (Kautto et al., 2017). The authors analyzed paired whole-exome sequencing (WES) data from 11,139 tumor-normal samples derived from either TCGA database, the TARGET45 database, or from other studies. In this tool, 2530 microsatellites loci were analyzed, containing only two of the loci assessed in both the Bethesda and Promega MSI-PCR panels, and neither of these were within the set of 22 loci that showed a significant different score in MSI-H versus MSS. MSI was detected in 27 of the 39 total types of cancer. They observed similar rates of MSI in the 18 type cancers as shown by Hause et al. The results showed a significant disease-specific prevalence of MSI, from 31.4% in EC to 0.25% in glioblastoma and the 3 cancer type with the highest rates of MSI prevalence were as expected EC, CRC and GC. Consistent with previous studies, MSI was more frequent in colon adenocarcinoma (19.7%) than rectal adenocarcinoma (5.7%). In addition, tumors other than these type were analyzed, and 0.8% were MSI-H. Of importance, MSI was detected in three cancer types that had not been previously characterized, adrenocortical carcinoma (MSI-H in 4.3%), cervical squamous cell carcinoma and endocervical adenocarcinoma (2.6%), and mesothelioma (2.4%). This study demonstrated a high sensitivity and specificity (97% and 99%, respectively) of MANTIS as compared with samples with known MSI status by MSI PCR across six cancer types, although the limitation of the study was related to the absence of data for MLH1 hypermethylation.

We have also evaluated 12,019 cancers, from 32 distinct tumor types for MMR deficiency using a NGS–based approach and found MSI-H frequency > 2% in 11 of these tumor types. As anticipated, the rate of dMMR cancers in stage I-III was higher than in stage IV cancer (Le et al., 2017).

Middha et al. reported the results of 13,091 cancer samples, including 66 metastatic cancer types sequenced with MSK-IMPACT (The Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets) an NGS clinical assay (Cheng et al., 2015; Hechtman et al., 2017). This represents the most extended series so far published. An MSIsensor score of 10 was determined as a sensitive cut-off to separate MSS from MSI-H tumors, which was validated in a cohort of CRC and EC. They found that 1.8%, (20 tumor types) displayed an MSI-H phenotype. Concordant with the Bonneville study, and as expected, MSI-H prevalence was higher among EC (15.6%) and CRC (8.3%). The inclusion of only metastatic disease, could explain the lower prevalence of MSI-H tumors in this cohort, as compared to other studies (Middha et al., 2017). Among the patients without CRC/EC, bladder cancer (3.1%), esophagogastric carcinoma (2.5%), and prostate cancer (1.7%) had the higher MSI-H incidence.

Overall, they observed a high sensitivity and specificity for MSI-H in CRC and EC (100% and 99.3%, respectively), as well as a high sensitivity and specificity across other tumor types (96.6% and 100%). Accordingly, all MSH6-deficient tumors were MSI-H on MSIsensor assessment, suggesting better performance in this subset of cancers (Stelloo et al., 2017). Finally, while survival difference according to MSI was confirmed in CRC in favor of MSI-H, no difference in other tumor types in term of survival were observed. In this case, an analysis of overall microsatellite burden as a continuous variable was not performed.

4. Frequency of MSI across human tumors

MSI occurs at different frequencies across malignancies and its signatures may differ among different cancer types: instability may concern different loci in different cancer types (Chang, Chang, Chang, & Chang, 2017). However, the majority of the data available to date come from examination of studies restricted to cancer types where clinical MSI testing was routinely performed (mostly CRC and EC) (de la Chapelle & Hampel, 2010; Kim et al., 2013).

Indeed, the prevalence of MSI in many other cancer types has been less described and significant between-study heterogeneity has been reported, as predictable given the small groups of patients in most of the studies, and the differences in methods used to evaluate MSI/dMMR, above all when considering those studies performed before the development of more standardized markers for MSI PCR testing.

In fact, although MSI has been studied for decades, the interest in investigating this molecular signature across a broad range of tumor types has emerged due to the US Food and Drug Administration's (FDA) recent approval of immunotherapy in MSI/dMMR advanced cancers and the large amounts of sequencing data now available (Diaz Jr. & Le, 2015; Le et al., 2015).

As noted in Table 1, MSI is a common phenomenon observed across different solid tumor types. Examples of common cancers that have MSI-H frequency > 10% include CRC, EC, and GC. Cancers with MSI-H frequency between 2% and 10% include ovarian cancer, cervical cancer, and thyroid cancer. Cancers with MSI-H frequency < 2% include prostate, non-small cell lung cancer (NSCLC), sporadic glioblastoma (GB) (Table 1).

5. Clinico-pathological feature of MSI/dMMR cancers

Microsatellite instability is overall observed in about 12% to 15% of CRC. The age distribution of MSI cancers follows a U-shaped distribution (Vilar & Gruber, 2010), with sporadic MSI cases generally diagnosed in older patients (>70 years) whereas hereditary cases are usually diagnosed in patients under the age of 50 (Shibata, 2002). MSI-H CRC, have a better prognosis in early stage disease (Gryfe et al., 2000; Popat, Hubner, & Houlston, 2005) and patients with LS-related MSI-H CRC have an increased risk of developing synchronous or metachronous colon cancer (Cai et al., 2003).

The majority of CRC is characterized by genetic instability due to an accelerated rate of gains or losses of whole or large portions arms of chromosome, resulting in chromosome number variability (aneuploidy), high frequency of loss of heterozygosity, chromosomal translocations or gene amplifications. This pathway of genetic instability is called chromosomal instability (CIN) and is present in ~60% of CRCs. CIN tumors are more common in the distal colo-rectum, and tend to show non-mucinous histology, moderate differentiation, and fewer tumor-infiltrating lymphocytes (TILs). Such tumors tend to arise from adenomatous polyps and appear to develop along the classic genetic pathway of colorectal tumorigenesis, with mutations in APC, KRAS and TP53, as first described by Fearon and Vogelstein (1990).

MSI-H CRC, by contrast, usually have near-diploid karyotypes, with preserved chromosomal architecture (Funkhouser Jr. et al., 2012) and its genetic instability is secondary to MSI due to a defective MMR system. However, a small proportion (<5%) of CRCs has both MSI and CIN, and their molecular features generally resemble those of MSI-H/CIN negative tumors (Trautmann et al., 2006). MSI cancers also often

Table 1
Frequency of MSI among different solid tumors.

Tumor type	Assay used	Frequency (%)	Reference
Colorectal cancer	MSI PCR MMR IHC Germline mutation MLH1 promotor hypermethylation	13%	Hampel et al. (2005)
	MSI PCR MMR IHC	17%	Ashktorab et al. (2016)
	NGS	19%	Hause et al. (2016), Bonneville et al. (2017)
		17%	Cortes-Ciriano, Lee, Park, Kim, and Park (2017)
		6%	Le et al. (2017)[a]
		8%	Middha et al. (2017)[b]
Endometrial cancer	MSI PCR MLH1 promoter hypermethylation	33%	Zighelboim et al. (2007)
	NGS	30%	Hause et al. (2016) and Bonneville et al. (2017)
		28%	Cortes-Ciriano et al. (2017)
		17%	Le et al. (2017)
Gastric cancer	MSI PCR MLH1 promotor hypermethylation	22%	Bass et al. (2014)
	MSI PCR MMR IHC	8%	Seo et al. (2009)
	NGS	3%[c]	Middha et al. (2016)
		8%	Le et al. (2017)
		21%	Cortes-Ciriano et al. (2017)
Rectal cancer	NGS	3%	Hause et al. (2016)
		6%	Bonneville et al. (2017)
		9%	Cortes-Ciriano et al. (2017)
Small Intestinal Malignancies	NGS	8%	Le et al. (2017)
Thyroid cancer (follicular and papillary)	MSI PCR	63%[d]	Mitmaker, Alvarado, Begin, and Trifiro (2008)
	NGS	2%	Le et al. (2017)
Hepatocellular carcinoma	MSI PCR MMR IHC	16%[e]	Chiappini et al. (2004)
	NGS	2–3%	Le et al. (2017), Cortes-Ciriano et al. (2017)
Ampullary carcinoma	MSI PCR MMR IHC	10%	Ruemmele et al. (2009)
Cholangiocarcinoma	NGS	2%	Bonneville et al. (2017), Le et al. (2017)
Skin cutaneous melanoma	MSI PCR	11%[f]	Palmieri et al. (2003)
	NGS	1%	Bonneville et al. (2017)
Ovarian cancer	MSI PCR MLH1 promotor hypermethylation	10%	Murphy and Wentzensen (2011)
	NGS	2–3%	Hause et al. (2016), Bonneville et al. (2017), Le et al. (2017) Cortes-Ciriano et al. (2017)
Cervical cancer	MSI PCR	7%	Lazo (1999)
	NGS	2–3%	Bonneville et al. (2017), Cortes-Ciriano et al. (2017), Le et al. (2017)
Pancreatic adenocarcinoma	MSI PCR	0%	Laghi et al. (2012)
		9%	Maple et al. (2005)
	NGS	1–2%	Cortes-Ciriano et al. (2017), Le et al. (2017)
Head and neck squamous cell carcinoma	MSI PCR	3%	Glavac, Volavsek, Potocnik, Ravnik-Glavac, and Gale (2003)
	NGS	1%	Hause et al. (2016), Bonneville et al. (2017), Cortes-Ciriano et al. (2017)
Renal clear cell carcinoma	MSI PCR MMR IHC	2%	Stoehr et al. (2012)
	NGS	1–2%	Hause et al. (2016), Bonneville et al. (2017), Cortes-Ciriano et al. (2017)
Prostate cancer	MSI PCR	1%	Burger et al. (2006)
	NGS	1–2%	Hause et al. (2016), Bonneville et al. (2017), Middha et al. (2017), Cortes-Ciriano et al. (2017), Le et al. (2017)
Bladder cancer	MSI PCR	1%	Catto, Xinarianos, Burton, Meuth, and Hamdy (2003)
	NGS	1%	Hause et al. (2016), Bonneville et al. (2017), Cortes-Ciriano et al. (2017)
Lung adenocarcinoma	MSI PCR	0%	Takamochi et al. (2017), Cortes-Ciriano et al. (2017)
	NGS	<1%	Hause et al. (2016), Bonneville et al. (2017), Le et al. (2017)
Lung squamous cell carcinoma	NGS	1%	Hause et al. (2016), Bonneville et al. (2017), Cortes-Ciriano et al. (2017)
Lung small cell cancer	NGS	1%	Le et al. (2017)
Breast invasive carcinoma	NGS	1–2%	Cortes-Ciriano et al. (2017), Hause et al. (2016), Bonneville et al. (2017)
Glioblastoma multiforme	NGS	1%	Hause et al. (2016), Bonneville et al. (2017), Cortes-Ciriano et al. (2017), Le et al. (2017)
Adrenal cortical carcinoma	NGS	6%	Cortes-Ciriano et al. (2017)
		4%	Bonneville et al. (2017)
Mesothelioma	NGS	3%	Bonneville et al. (2017)
Uterine sarcoma	NGS	2%	Le et al. (2017)

IHC, immunohistochemistry; MMR, mismatch repair: MSI, microsatellite instability; PCR, polymerase chain reaction.
[a] More patients in stage IV evaluated.
[b] Study conducted only in the metastatic setting.
[c] Including esophagogastric patients.
[d] Only 23 patients evaluated.
[e] Only 37 patients in total evaluated.
[f] Evaluation of 56 primary melanoma, using a panel of 13 polymorphic microsatellites.

exhibit a CpG island methylator phenotype (Samowitz, 2007; Weisenberger et al., 2006).

MSI CRC tend to arise from sessile serrated adenomas in the proximal colon (Aaltonen et al., 1993) including the transverse colon, (Cai et al., 2003; Thibodeau, Bren, & Schaid, 1993) which is part of the embryologically derived midgut, and are rare in the hindgut derived descending, sigmoid colon and rectum (Sinicrope, Rego, Foster, et al., 2006). Other pathologic differences exist: MSS CRC typically show infiltrating glands with dirty necrosis while MSI tumors are characterized by TILs and the Crohns-like lymphocytic host response, poor differentiation with pushing margins, and mucinous differentiation (Greenson et al., 2003). Mucinous differentiation is also the most common histology found in dMMR ECs.

It is of interest that histologic subtypes of MSI-H ovarian cancer revealed an overrepresentation of non-serous subtypes (mucinous and endometrioid) (Pal, Permuth-Wey, Kumar, & Sellers, 2008).

GCs with MSI-H have distinct clinical and molecular features compared to MSS GC, and share common characteristics with CRC MSI-H patients, as described above. Indeed, sporadic MSI-H GC are associated with older age and female sex, they usually are located in the gastric antrum, present with well differentiated or intestinal-type histology, and are diagnosed at earlier stage, harboring a smaller risk of lymph node metastasis, and a better prognosis (Seo et al., 2009). Association with tumor necrosis, expanding growth pattern, and TILs are also reported (Mathiak et al., 2017).

5.1. DNA MMR status as prognostic/predictive biomarker

5.1.1. Prognostic implications

Despite poor histologic differentiation, the biological behavior of MSI-H CRC is less aggressive compared to that of MSS CRC, and is associated with a lower stage at diagnosis and improved stage-specific prognosis, although other studies have suggested that any favorable prognostic effect of dMMR is limited to patients with earlier-stage tumors (Kim et al., 2007).

Gryfe et al. reported the first cohort of patients demonstrating the favorable prognostic value of MMR deficiency in CRC. Authors showed that MSI-H tumors had a more favorable prognosis, with a lower tendency to lymphnode spread and metastasis development, compared to the stable counterpart (Gryfe et al., 2000). Other studies have confirmed these results and observations. Mouradov et al. showed that MSI and CIN are independent markers of disease free survival (DFS) in stage II/III CRC, with MSI-H indicating good prognosis and CIN+ poor prognosis (Mouradov et al., 2013). This was confirmed in other studies (Sinicrope, Rego, Halling, et al., 2006; Watanabe et al., 2012). In a retrospective analysis, MSI-H phenotype was associated with significantly improved relapse free survival among tumors in the proximal colon (Hazard ratio (HR) 0.71; 95% CI: 0.53–0.94; $P = 0.018$), but not in the distal after adjustment for KRAS and BRAF V600E mutations and other relevant prognostic covariates (Sinicrope, Rego, Halling, et al., 2006). Intriguingly, the beneficial effect in stage III MSI CRC patients appeared to be limited to CRC harboring germline mutations in MMR genes (Tejpar, Saridaki, Delorenzi, Bosman, & Roth, 2011).

Two recent metanalysis have confirmed these findings. A large series of 2935 stage II or III CRCs found better overall survival (OS) for MSI-H or dMMR (HR 0.67, 95% CI 0.58–0.78) (Popat et al., 2005). Guastadisegni et al. found that MSI-H was associated with better OS (HR 0.6; 95% CI: 0.53–0.69; $P < 0.001$) and better DFS (HR 0.58; 95%CI: 0.47–0.72; $P < 0.001$) when compared to MSS (Guastadisegni, Colafranceschi, Ottini, & Dogliotti, 2010).

These conclusions have been accompanied by clinical observations. First, the prevalence of MSI-H CRCs is different among disease stages. Indeed, while dMMR/MSI-H CRCs represent 15–20% of stage II and III CRCs, they represent only about 4% of metastatic CRC (mCRC) cases. This lower frequency highlights the weakened capacity for dMMR CRCs to develop metastasis. However, dMMR CRC may carry a worse prognosis than pMMR CRCs in the metastatic setting, and this has been in part explained by the opposite prognostic effect harbored by BRAF mutation, which are present in higher rates in dMMR mCRCs (35%) compared with early-stage dMMR CRCs (24%) (Lochhead et al., 2013). Indeed, there is evidence of the negative prognostic effect of BRAF mutations in CRC, but the entire picture is more complex, since BRAF mutation is also strongly associated with MSI phenotype, which is an indicator of good prognosis. Different studies sought to determine if the BRAF V600E mutation may provide additional prognostic value when evaluated together with MMR status. Unfortunately, studies have reached conflicting results, when looking at different populations (stage II-III versus stage IV CRC). French et al. examined 533 tumors from high-risk stage II or stage III CRC patients enrolled in a randomized prospective clinical trial. The MMR status was defined by IHC stain coupled with PCR testing for MSI. In this study, they confirmed that MMR status is an important prognostic marker for CRC patients, with a magnitude of effect consistent with that previously observed (15% increase in 5-year DFS and OS). Importantly, they showed that the concomitant evaluation of both the MMR status and BRAF mutation status in CRC provided more prognostic information than either factor alone. Patients whose tumors were BRAF wild type/dMMR have a significantly improved OS as compared to BRAF mutated/dMMR CRC (French et al., 2008). However, further studies demonstrated that the positive prognosis impact of MSI, could overcome the negative effect of BRAF mutation. In the study by Hutchins et al., no significant difference in risk of recurrence was found based on BRAF mutation status, in stage II and III MSI-H CRC (Hutchins et al., 2011), and the same conclusion were drawn by Samowitz et al., in stage IV CRC patients, where they showed that MSI-H CRC had an excellent 5-year survival regardless of the BRAF status (Samowitz et al., 2001). Phipps and colleagues investigated the association between mutation profile and MMR status in a large population-based registry cohort of CRC patients and found a significantly better outcome (measured as DFS and OS) in MSI-H, BRAF mutated CRC patients as compared to BRAF mutated MSS tumors (Phipps et al., 2016).

The positive prognostic effect of MSI-H has been suggested in several other tumor types (gastric, ovarian, upper urinary tract urothelial cancer, biliary tract cancers) although controversial results have been reported, and most of these are retrospective studies, lacking for a control group (Campanella et al., 2017; Cloyd et al., 2017; Ikoma et al., 2017; Kato et al., 2015). For instance, Hause et al., provided evidence that increasing MSI positively correlates with survival time in different MSI cancers, data not confirmed by MSK IMPACT outside of the CRC cohort (Hause et al., 2016).

5.1.2. Influence on response to chemotherapy

Components of the MMR machinery bind to 5-fluorouracil (5-FU) incorporated DNA, contributing to the observed cytotoxic response. These findings supported the hypothesis that MSI predicts absence of benefit from 5-FU-based adjuvant chemotherapy (Barratt et al., 2002). Ribic et al. in 2003 were among the first to show the benefit of 5-FU–based adjuvant chemotherapy in stage II or III MSS CRC patients but not in MSI-H patients (Ribic et al., 2003), with even a suggestion of harm in term of OS in treated CRC with MSI-H tumors. Further clinical studies have substantiated the finding that MSI patients do not benefit from 5-FU therapy, with some exceptions (Tejpar et al., 2011). Sargent et al. in a pooled analysis of a total of 1027 patients, observed a statistically significant improved DFS ($P = 0.001$) in patients with stage III MSS tumors receiving adjuvant 5-FU chemotherapy, but no treatment effect in stage III MSI-H tumors. Notably, a non-statistically significant benefit of adjuvant therapy was observed in patients with stage II MSS tumors, whereas they reported reduced DFS and OS in treated MSI-H stage II patients compared with non-treated MSI-H controls (Sargent et al., 2010). Sinicrope et al. reached the same conclusions reporting statistically significant benefit of 5-FU treatment in stage III patients with MMR germline (versus sporadic) mutations (TTR, $P = 0.016$; DFS, $P = 0.047$; OS, $P = 0.041$) but not in stage II (Sinicrope et al., 2011). As a

consequence, MMR protein status assessment is recommended by the NCCN and the European Society for Medical Oncology guidelines for patients with resected stage II CRC for consideration of adjuvant chemotherapy (National Comprehensive Cancer Network, 2018; Van Cutsem et al., 2016).

The impact of MMR status remains controversial in the era of the standard FOLFOX adjuvant chemotherapy for stage III CRC. A post hoc analyses of patients with stage II and III CRC (n = 1796) from NSABP-C07 and NSABP-C08 trials suggested that the benefit of adding oxaliplatin was independent of MMR status (Gavin et al., 2012). The same conclusion was provided by the analysis from the MOSAIC trial, which showed that patients with both dMMR and pMMR stage III tumors had a survival benefit from FOLFOX compared with fluorouracil alone (Andre et al., 2015).

A resistance to 5-FU–based chemotherapy in dMMR GC has also been reported. Smyth et al. recently examined the association among MMR deficiency/MSI-H and survival in patients with resectable gastroesophageal cancer randomized to surgery alone or perioperative epirubicin, cisplatin, and fluorouracil. In this exploratory analysis, they found that patients with operable MSI-H gastroesophageal cancer had superior survival compared with patients with MSI-L or MSS tumors when treated with surgery alone. However, patients with MSI-L or MSS tumors had superior survival compared to the MSI-H ones when treated with perioperative chemotherapy, hence suggesting that MSI-H gastroesophageal cancers did not benefit from perioperative chemotherapy (Smyth et al., 2017). If validated, this finding has the potential to improve patient selection for perioperative chemotherapy based on MMR status.

Preclinical studies have suggested that MSI CRC cell lines are more sensitive to irinotecan compared with MSS counterparts. For instance it has been reported that MSI-H CRC cell lines harboring mutations in microsatellites located in an intron–exon boundary polyt(11) repeat in MRE11A and in a coding polya(9) tract in hRAD50, show a particularly high sensitivity to irinotecan. However, since MMR deficiency does not always result in MRE11A or hRAD50 mutations, with mutations in these genes detected in <70% of MSI-H tumors, MSI is not an ideal predictive marker for irinotecan based therapy (Magrini et al., 2002; Pommier, 2006; Vilar et al., 2008). Few clinical studies have analyzed the activity of irinotecan in MSI-H CRC, and the results are controversial and inconclusive. A prospective analysis of 702 stage III CRC patients included in the CALGB protocol 89,803 study, evaluated the efficacy of irinotecan, 5FU and folinic acid compared with a weekly bolus of 5-FU as adjuvant therapy, and showed a trend toward a benefit of MSI-H tumors treated with the combined regimen in terms of 5 year DFS (Bertagnolli et al., 2009), albeit a subsequent retrospective analysis from PETACC3 trial did not confirm these results (Tejpar et al., 2011). As for stage IV CRC patients displaying MSI-H, a meta-analysis was attempted to address this question, but unfortunately, but due to lack of enough power, authors were unable to achieve any conclusion about the role of irinotecan-based regimens in MSI tumors.

In regard to EC, Resnick et al. reported that subgroup of patients with non-endometrioid EC and dMMR had improved survival after adjuvant radiotherapy, suggesting that dMMR status might provide predisposition to be sensitive to adjuvant radiotherapy (Resnick et al., 2010). In a previous study, although not significant, responses to platinum-based chemotherapy was higher in dMMR EC as compared with pMMR patients. However, a recent large meta-analysis pooling 23 studies concluded for lack of concrete evidence of association between dMMR status and clinical outcome in platinum-treated EC (Diaz-Padilla et al., 2013). Recently Heby et al. showed that dMMR was associated with a significantly prolonged OS in a cohort of 172 periampullary adenocarcinomas (HR = 0.32, 95% CI 0.17–0.61), although not independent of conventional prognostic factors. Interestingly, in the pancreatobiliary tumor subgroup, dMMR was only prognostic in non-adjuvant treated cases (HR = 0.26, 95% CI 0.08–0.85), while there was a significant negative interaction between dMMR and adjuvant treatment (Heby et al., 2018). These findings corroborate the urgent question about the potential predictive role for MMR status for sensitivity or resistance to therapies.

In summary, the value of MMR status as a predictive marker of response to 5-FU, irinotecan and other chemotherapeutic agents remains controversial. First, the stratification of patients in subgroups according MMR status makes the sample sizes too small to show a different effect of chemotherapeutic agents; second, the results available may have been affected by the retrospective and/or single institutional type of most of the studies cited, with different methods used to assess MSI.

However, in the era of personalized medicine, a more standardized assessment of dMMR/MSI-H phenotype in prospective and adequately powered studies, may provide valuable information, which may eventually be of predictive and therapeutic utility.

6. dMMR/MSI tumors and increased neoantigen burden and response to PD-1 blockades

Inactivation of MMR leads to the MSI-H phenotype, characterized by a high frequency of insertions/deletions due to unrepaired DNA polymerase slippage in microsatellites sequences (Arana & Kunkel, 2010; Drake et al., 1998). Genes most commonly targets of frameshift mutations caused by MSI are involved in different cellular functions, such as DNA repair (MSH3 and MSH6, MRE11A), epigenetic regulation (HDAC2, ARID1A), cell signaling (TGFBR2, IGFR2, ACVR2A), apoptosis (BAX), and miRNA processing (TARBP2, XPO5). There is evidence of a tumor-type specificity of frameshift MSI (Muzny et al., 2012; Nebot-Bral et al., 2017). For instance, frameshift mutations in TGFBR2 are more common in CRC and GC (58% and 80% respectively) but only present in 5% of ECs. On the other hand, frameshift mutations in the JAK1 gene have been reported in both ECs and CRCs (about 20%) (Cortes-Ciriano et al., 2017). This is important, given that JAK1 mutations have been shown to be involved in the resistance to immunotherapy in melanoma (Shin et al., 2017).

A subset of these insertions and deletions can affect coding regions of the genome and generate frameshifts in the open reading frame of genes, thus resulting in the production of truncated, functionally inactive, proteins and generate neoantigens that are qualitatively different from self (Linnebacher et al., 2001; Saeterdal et al., 2001; Schumacher & Schreiber, 2015). Endogenous cytotoxic T-lymphocytes (CTLs) can recognize these neoantigens that are displayed on major histocompatibility complex (MHC) I molecules at the surface of tumor cells, thus increasing the TILs density and triggering an immune response in the host (Segal et al., 2008; Yarchoan, Johnson III, Lutz, Laheru, & Jaffee, 2017).

In addition to MSI, dMMR tumors are also characterized by high rate of single-nucleotide substitutions.(De Grassi et al., 2010). In CRC for instance it has been showed that approximately 1300 somatic base substitutions are acquired in MSI-H LS related CRC while only 190 somatic base substitutions are present in MSS tumors (Campbell et al., 2017; Muzny et al., 2012; Timmermann et al., 2010).

Recent progress in genomic analysis using WES and NGS technology has enabled comprehensive detection of mutations and mutation burden in cancer tissues (Table 2), revealing an higher average of somatic mutations (many of which predicted to result in neoantigens) in MSI-H cancers compared to MSS cancers. In the study by Middha et al., only three of 10,900 patients with dMMR/MSI-H status did not display a high tumor mutation burden (TMB) (Hechtman et al., 2017). In the MANTIS study, the mean of somatic mutations, both nonsynonymous and synonymous, was found to be constantly increased among MSI-H versus MSS tumors within all the tumor types (Kautto et al., 2017). Greenman et al. reported the sequencing of 518 protein kinase genes in 210 diverse human cancers, demonstrating a mutation rate for dMMR tumor approximately 25-fold higher than in proficient tumors (Greenman et al., 2007). One of the largest series investigating the relationship between TMB and MMR status, has been published recently by Chambers et al., Using targeted comprehensive genomic profiling essay

Table 2
Current clinical trials of immune-based therapy in dMMR/MSI-H solid tumors in adult and pediatric populations.

Immunotherapy drug (mechanism of action)	Study treatment design	Phase	Population included	Indication	Recruitment status	Ref.
Atezolizumab (anti-PD-L1)	Fluorouracil, oxaliplatin, and leucovorin calcium (mFOLFOX6)/bevacizumab ± Atezolizumab IV	III	Stage IV dMMR/MSI-H CRC	First line	Recruiting	NCT02997228
	mFOLFOX6 ± Atezolimumab IV	III	Stage III, dMMR/MSI-H CRC	Adjuvant therapy	Recruiting	NCT02912559 (ATOMIC)
	Atezolizumab Bevacizumab	II	Stage IV, MSI-like CRC	Chemotherapy resistant	Recruiting	NCT02982694 (COMMIT)
Pembrolizumab (anti-PD1)	Arm A: pembrolizumab Arm B: FOLFOX or FOLFIRI (±cetuximab or bevacizumab)	III	Stage IV, dMMR/MSI-H CRC	First line	Closed to enrollment	NCT02563002 (KEYNOTE-177)
	Preoperative pembrolizumab IV followed by postoperative pembrolizumab + capecitabine and radiation therapy	II	Resectable GC: • dMMR/MSI-H • EBV+	Perioperative	Recruiting	NCT03257163
	Pembrolizumab IV, followed by taxane-based chemotherapy at the time of progression	II	mCRPC: • Group A: DNA damage repair[a] proficient • Group B: DNA damage repair[a] deficient	mCRPC	Not yet recruiting	NCT03248570
	Pembrolizumab	II	Advanced solid tumor, including dMMR/MSI-H CRC	Progression or intolerance to standard therapies	Recruiting	NCT02628067 (KEYNOTE-158)
	Pembrolizumab	II	• MSI CRC • MSI non CRC • MSS CRC	≥2 prior therapies	Recruiting	NCT01876511 (KEYNOTE-016)
	Pembrolizumab Itacitinib (JAK1 inhibitor)	I	Advanced solid tumors, including MSI CRC, bMMRD tumors	Refractory to standard therapy	Recruiting	NCT02646748
	Pembrolizumab Epacadostat (IDO-inhibitor)	I/II	Advanced solid tumor including EC, MSI-H CRC, GC	>1 prior therapy	Recruiting	NCT02178722 (KEYNOTE-037)
	Pembrolizumab	I	• bMMRD- or LS – related brain tumors • Malignant Glioma • Diffuse Intrinsic Pontine Glioma	Refractory or recurrent after standard therapy	Recruiting	NCT02359565
Avelumab (anti-PD-L1)	Avelumab	II	Stage IV CRC: • dMMR/MSI-H • POLE mutated	>1 line therapy	Recruiting	NCT03150706
	Arm A: FOLFOX/FOLFIRI ± targeted therapy Arm B: Avelumab	II	Stage IV, MSI CRC	Refractory to standard therapy	Not yet recruiting	NCT03186326 (PRODIGE 54)
	Avelumab		• MSS EC • MSI-H EC • POLE mutated EC	Refractory or recurrent after standard therapy	Recruiting	NCT02912572
Durvalumab (anti-PD-L1) + AZD9150 (STAT3 inhibitor)	AZD9150 every week + MED4736 every 4 weeks	II	• NSCLC • Pancreatic Cancer • dMMR/MSI-H CRC	Refractory to standard therapy	Recruiting	NCT02983578
Nivolumab ± other immunotherapy agents	Cohort 1:Nivolumab Cohort 2: Nivo + Ipi (escalation dose) Cohort 3: Nivo + Ipi Cohort 4: Nivo + Ipi + Cobimetinib Cohort 5: Nivo + BMS-986016 Cohort 6: Nivo + Daratumumab	II	Stage IV CRC: • MSI/dMMR • MSS/pMMR	Recurrent or metastatic	Recruiting	NCT02060188 (CHECKMATE 142)
	Nivolumab + Ipi + Radiation Therapy	II	• MSS CRC and Pancreatic cancer • MSI-H CRC	Refractory or recurrent to standard therapy	Recruiting	NCT03104439
	Nivolumab	II	• RCC • Head and neck neoplasm; • Skin neoplasms • MSI non CRC • Penile neoplasms	Resistant or refractory to standard therapy	Not yet recruiting	NCT03012581
		I-II	• Hypermutated malignancies in bMMRD	Refractory or recurrent after standard therapy	Active not recruiting	NCT02992964
Ly3300054 (anti-PD-L1)	Ly3300054 Ramucirumab (anti-VEGF) Abemaciclib (CDK4/6inhibitor) Merestinib (c-MET inhibitor)	I	• MSI-H solid tumors • Cutaneous melanomas	Advanced, refractory	Recruiting	NCT02791334
APX005M (CD40 agonist)	APX005M	I	• Urothelial carcinoma • MSI-high tumors • Other solid tumor types	After standard therapy	Recruiting	NCT02482168

bMMRD, biallelic mismatch repair deficiency; CRC, colorectal cancer; dMMR, deficient DNA mismatch repair; EBV, Epstein Barr Virus; Ipi, ipilimumab; mCRPC, metastatic castration resistant prostate cancer; MSI, microsatellite instable (MSI); MSS, microsatellite stable; Nivo, nivolumab; NSCLC, non-small cell lung cancer; pMMR, proficient DNA mismatch repair; Ref., references.
[a] Including DNA mismatch repair (MMR).

on 62,150 cancer samples, authors found that the vast majority (83%) of MSI-H samples had high TMB, with 97% displaying >10 mutations/Mb (Chalmers et al., 2017).

The clinical significance of identifying hypermutated tumors has recently been demonstrated by several studies showing that TMB, and consequent mutation-associated neoantigens (MANA) load, suits as a promising predictive biomarker of benefit for ICIs therapy (Diaz Jr. & Le, 2015; Rizvi et al., 2015; Schumacher & Schreiber, 2015). Indeed, given the promise that immune-based therapies, and ICIs specifically, have shown in treatment of refractory disease and the durable responses observed, there is great interest in identifying patients who are most likely to derive benefit from these therapies (Fig. 1) (Jin & Yoon, 2016; Nebot-Bral et al., 2017; Rizvi et al., 2015).

In preclinical models of CRC, Bardelli et al., showed that in dMMR tumors, not only there was a higher mutational load, but also the number of predicted neoantigens evolved dynamically over time, supporting that DNA MMR inactivation promotes the continuous emergence of neoantigens (Germano et al., 2017).

In our trial, we observed that MSI-H patients with high number of somatic mutations, resulting in higher MANAs load, had a longer progression free survival (PFS) and trend toward a better objective response to pembrolizumab. Importantly, all the MANAs identified resulted from frameshift mutations. Through deep sequencing of T cell receptor CDR3 regions to evaluate T cell clonal in both tumors and blood and through testing for their reactivity against candidate MANAs, we provided also evidence that dMMR tumors harbor functional MANA-specific T cells that are peripherally expanded by checkpoint blockade in responders (Le et al., 2017).

Other in vitro and in vivo studies confirmed that T helper cells and CTLs are exquisitely specific for neoantigen produced from frameshift mutations in dMMR tumors and that the amount of these frameshift neoantigens positively correlates with higher density of TILs, as already shown in CRC but also in other tumor types. For instance, Lee et al. found that MSI-H ECs have 7-fold higher neoantigens levels in comparison with MSS cancer and the number of CD3+ and CD8+ cells invading cancer tissues is also significantly higher ($P = 0.001$ and $P < 0.001$, respectively) (Lee, Kwak, et al., 2017; Lee, McNulty, Duncavage, Heusel, & Hagemann, 2017). Recently, a study by Nakamura on 260 biliary tract cancers, found 14 hypermutated tumors (mutation rates of >11.13/Mb). Of these, five harbored inactivating mutations in MMR proteins. Transcriptome sequencing and hierarchical clustering of gene expression levels showed that hypermutated tumor demonstrated significant enrichment for immunomodulatory pathways, with an higher expression of immune checkpoint molecules, upregulation in genes involved in cytokine activity and interferon signaling, supporting that this subgroup may be a good target population for immunotherapy (Nakamura et al., 2015).

However, lessons learned from the past, make it highly unlikely that clinical responders to ICIs will be identified by a single biomarker, such as dMMR, given the complex biology of tumors and the immunological cascade that must be triggered in an efficacious anti-tumor immune response. At present there is no clear strategy to establish what these

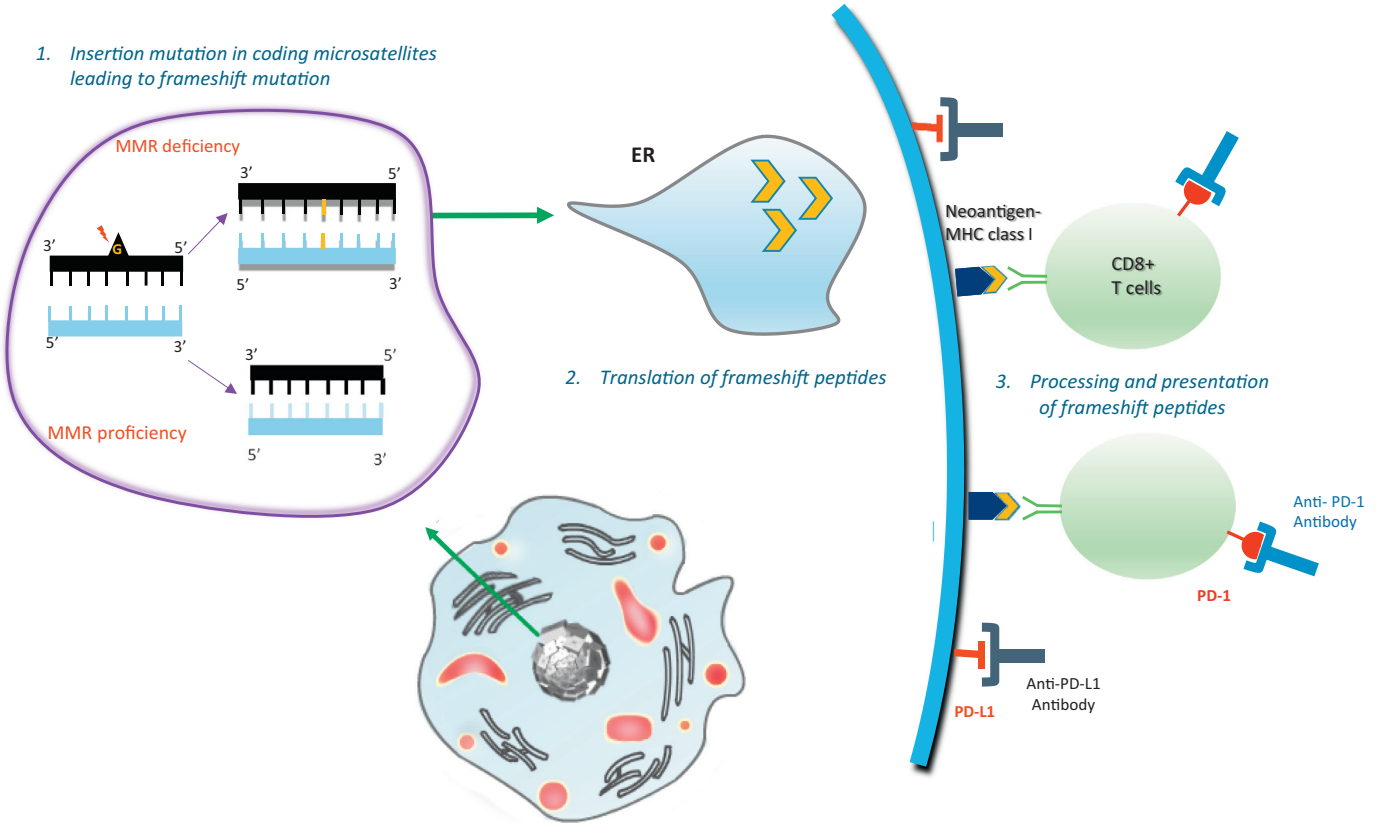

Fig. 1. Frameshift mutation produced by deficit in MMR system, generate neoantigens and elicit immune response, further enhanced by immune checkpoint blockade. Inactivation of MMR leads to the MSI phenotype, characterized by a high frequency of insertion/deletions due to unrepaired DNA polymerase slippage in microsatellites sequences. When these insertions and deletions affect coding regions, they can result in frameshift mutations and generate neoantigens. These neoantigens arise as a by-product when frameshifted neoproteins are degraded and derived peptides are presented. Neoantigens are therefore individual, immunogenic peptides, non-self for the immune system and unique to the tumor. Endogenous cytotoxic T-lymphocytes (CD8+ T cells) can recognize these neoantigens that are displayed on MHC I molecules at the surface of tumor cells, thus increasing the TIL density and triggering an immune response in the host. This active immune microenvironment is counterbalanced by upregulation in several immune checkpoint ligands in the dMMR tumor microenvironment, including PD-1 and PDL1, which allow tumors to escape immune surveillance. Anti-PD-L1 and anti-PD-1 antibody bind to PD-L1 and PD-1, respectively, thus blocking immune tolerance resulting in an enhanced antitumor effect.

biomarkers should be and many questions remain open, as well as, important caveats should be acknowledged: 1) The relationship between TMB and MSI is imperfect. In the study by Chambers et al., only 16% of samples with high TMB were classified as MSI-H. Indeed, while in CRC, EC, GC MSI-H can be considered a surrogate marker for high TMB, in other tumor types (such as melanoma, and lung carcinoma) high TMB is commonly seen in the absence of MSI-H signature. Various other hypermutation signatures (POLE signature, UV exposure, smoking, temozolomide exposure (Hunter et al., 2006) have been shown to be associated with an increased mutation rate. 2) It remains still unclear what frequency of somatic mutations correlate with the generation of reliable and targetable neoantigens and ultimately what percentage of human cancers contain candidate neoantigens for successful immune targeting.

7. Impact of MMR on the tumor microenvironment (TME): The interplay between cancer genetics and cancer immunology

Activation of antigen-specific T cells is a key step in immune responses, and it is provided by the interaction between the peptide-MHC complex and the T cell receptor in the presence of other co-stimulatory molecules, such as cluster of differentiation 28 (CD28), expressed on the surface of naive CD4+ and CD8+ cells (Suh et al., 2004). However, during tumor equilibrium or progression, T-cells become exhausted, with overexpression of inhibitory receptors, especially, among others, programmed cell death 1 (PD-1, CD279), cytotoxic T lymphocyte antigen-4 (CTLA-4, CD152), lymphocyte-activation gene-3 (LAG-3), T-cell immunoglobulin domain and mucin domain-3 (TIM-3), IL-10 receptor, (Cao et al., 2007; Hathcock et al., 1993; Nishimura, Nose, Hiai, Minato, & Honjo, 1999; Zhang, Chikina, et al., 2017; Zhang, Sun, et al., 2017; Zou & Chen, 2008). CTLA-4, a CD28 homologue, competes with CD28 for binding to its cognate ligands, CD80/86, which are expressed on the surface of antigen-presenting dendritic cells (APCs) (Gardner, Jeffery, & Sansom, 2014). CTLA-4 acts earlier in the process of T-cell activation, in contrast PD-1 molecule plays a role later in the response after activation, by attenuating T-cell responses following migration of T cells to the TME, rendering T-cell dysfunctional and maintaining the exhausted T cell phenotype (Topalian, Drake, & Pardoll, 2012). Thereby PD-1 signaling limits the activity of T cells during inflammatory responses in order to prevent excessive host tissue damage (Naboush, Roman, & Shapira, 2017). This signaling is activated by its ligands, programmed cell death ligand (PD-L) 1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273). While PD-L2 is exclusively expressed on activated dendritic cells and macrophages, PD-L1 has a broad tissue distribution including tumor cells and is induced by inflammatory mediators (e.g. IFN-c, lipopolysaccharides, GM-CSF, IL-4 and IL-10) (Dong et al., 2002).

A rapidly increasing body of evidence demonstrates the interdependence of DNA MMR and immune microenvironment. Here we provide a picture of the correlation between MMR status, PD-L1 expression and TME characteristics in CRC, EC and GC, with some insights on how to potentially translate these findings into clinical practice.

7.1. CRC

High numbers of TILs represent a common hallmark of MSI CRC and it is a validated stage-independent predictor of increased survival in patients with CRC. dMMR/MSI-H CRC naturally attract TILs through the upregulation of the integrin molecule CD103 on CD8+ T cells. Moreover, a higher expression of immune activating molecules as well as increased expression of co-stimulatory molecules in tumoral DC, which serves for a proper T cell activation, and a dense CD4+ T cell infiltration is also reported in MSI-H but not MSS CRC. This is consistent with finding from gene expression profiles analysis consistently showing an upregulation of genes linked to immune responses and interleukin pathways (Muzny et al., 2012).

Llosa and colleagues recently refined these observations demonstrating that the active immune microenvironment in MSI-H CRC is counterbalanced by immune inhibitory signals. Indeed, they demonstrated an upregulation in several immune checkpoint ligands, including PD-1, PDL1, CTLA- 4, LAG-3, FOXp3 and Indoleamine 2,3-dioxygenase-1 (IDO1) (Llosa et al., 2015).

Some other studies reported higher PD-L1 expression in dMMR CRCs than in pMMR ones (Gentles et al., 2015; Lee et al., 2016; Lee, Kwak, et al., 2017; Lee, McNulty, et al., 2017; Li et al., 2016). However other authors have reached different conclusion (Masugi et al., 2017). This heterogeneity can be explained the different tumor compartments expressing PD-L1 studied (tumor cells versus TILs). Llosa et al. were among the first to demonstrate that in MSI-H CRC, the PD-L1 expression appears not to be on tumor cells, as commonly seen in melanoma or NSCLC, but on TILs and/or myeloid cells (Llosa et al., 2015). Subsequent studies have confirmed these finding. A recent study analyzing only stage I–III CRC patients demonstrated that dMMR CRC have an increased density of CD3+ ($P < 0.01$), CD45R0+ ($P < 0.05$) and CD8+ ($P < 0.071$) T lymphocytes positively associated with survival (Park & Cheung, 2017). Zhang et al. showed that the number of CD8+ T cells in the stroma and in the invasive front, but not within the tumor, was greater in the dMMR group than in the pMMR ($P = 0.017$ for TIL and stroma; $P = 0.038$ for invasive front) (Zhang, Chikina, et al., 2017; Zhang, Sun, et al., 2017). Le Flahec et al. analyzed the immune environment in dMMR and pMMR CRCs, considering all stages, and they also showed that immune cells (CD3+ and CD8+) were significantly more numerous in the stroma and at the invasion margin in dMMR tumors (Le Flahec et al., 2018). Discrepant data have been reported about the expression of regulatory T cells (Tregs). Tregs may inhibit anti-tumor immune responses, by suppression of CTLs and a higher ratio of CD8+ T cells to Tregs correlates with better outcome in CRC. Although not statistical significant, Llosa et al., observed higher Foxp3 + cell infiltrates, representative of Treg, in tumor stroma and the invasive front in MSI-H compared with MSS tumors (Llosa et al., 2015). Opposite results were reported by other studies (Le Gouvello et al., 2008; Maby, Galon, & Latouche, 2016).

7.2. EC

Data available are consistent with finding in CRC and showed that dMMR EC are characterized by a greater numbers of TILs and increased neoantigen production generated by frameshift mutations acquired from MSI, as well as an increased PD-L1 expression (Sloan, Ring, Willis, Modesitt, & Mills, 2017).

In this regard, one of the largest series has been published by Howitt et al., which showed a median neoantigen load per sample of 541 in MSI tumors versus 70.5 in MSS tumors. Similarly, MSI ECs had significantly higher numbers of CD3+ and CD8+ TILs compared to MSS ECs. However, this positive feature were counterbalanced by PD-1 and PD-L1 overexpression in intraepithelial immune cells and peritumoral lymphocytes ("marginal" or "infiltrating edge" pattern of expression) with no cases of extensive membranous tumor cells staining among MSI cancers (Howitt et al., 2015). Sloan et al. confirmed these findings (Sloan et al., 2017). Loss of MSH6 IHC expression and germline confirmed MSH6 mutations were particularly associated with tumoral PD-L1 expression. This is notable in EC, where MSH6 mutations account for a higher proportion of germline mutations compared with CRC.

7.3. GC

Kim et al. evaluated the expression status of PD-L1 in MSI-H and MSS GC samples based on four different cut-off values (1%, 5%, 10%, and 50%), and found a higher expression of PD-L1 in tumor cells and immune cells in MSI-H GCs (Kim et al., 2017). Other studies yielded corroborative results, demonstrating that PD-L1 positivity was more frequent in MSI-H than in MSS GC, either in tumor cells or in TILs along with a strong

association between PD-L1 expression and high densities of TILs (CD8+, CD3+, FOXp3+ and CD4+ T cells).

These studies have answered some crucial questions but have also raised new ones. First, it has recently been reported that PD-L1 expression in frequently discordant between surgically resected and matched biopsy specimens (the overall discordance rate in the study was 48%), primarily due to the lack of PDL1-positive TILs components in matched biopsies (Ilie et al., 2016), thus spatial heterogeneity could result in significant sampling bias and should be taken into account.

Second, a lot of discrepancies exist in term of IHC assay to analyze PD-1/PD-L1 expression, the cutoff used to consider it positive, as well as the assay used to evaluate MMR status, making it difficult a comparison among different studies.

However, important lessons can be learned by these observations. Although MSI-H tumors have an increased number of TILs, and a higher expression of immune checkpoint molecules, as consequence of many immunogenic neoantigens produced by frameshift mutations, mounting evidence exist that not all dMMR harbor dense infiltration of TILs, nor all express a high level of PD-1/L1. The biological meaning of these differential expression patterns is still unknown and might imply an exquisitely complex interplay involving both genetic and immunological variables and further studies are needed to better define the impact of MMR on the tumor immune contexture.

8. The genomic landscape of cancers shapes response to anti-PD-1 therapy

After many decades of development, immunotherapy is becoming a pillar of cancer therapy (Pardoll, 2012), with the great promise to prevent recurrence and prolong survival via the long-term memory function of the adaptive immune system (Brahmer et al., 2012; Le et al., 2015; Topalian, Hodi, et al., 2012).

In MSI-H tumors highly increased mutation rates and expression of immunogenic frameshift neopeptides set the stage for this responsiveness. In fact, these features positively coincide with extensive infiltration of the tumor by activated neoantigen-specific cytotoxic and T helper cells, resulting in an anti-tumor immune response. However, as showed above, the tumor itself creates an immunosuppressive microenvironment that blocks immunological action despite this recognition (Llosa et al., 2015). Reversal of this negative control with ICIs is an alternative approach to elicit efficacious immune responses to cancer.

The first prospective evaluation of dMMR/MSI-H as a predictive biomarker for PD-1 inhibition was reported in 2015 (KEYNOTE-016). The first results were reported on 11 patients with dMMR CRC, 21 with pMMR CRC, and 9 with dMMR non-CRC (4 ampullary or cholangiocarcinomas, 2 ECs, 2 small bowel carcinomas, and 1 GC) treated with pembrolizumab, a humanized monoclonal immunoglobulin (Ig) G4 antibody, and showed a significant difference in clinical response, based on MSI status, with 40% and 71% in overall response rate (ORR) in dMMR CRC and dMMR non-CRC, versus 0% in pMMR CRC, as well as in the PFS rate (78%, 67%, and 11%, respectively) (Le et al., 2017). In May 2017, the FDA granted accelerated approval to pembrolizumab for refractory, adult and pediatric, dMMR/MSI-H tumors including dMMR/MSI-H metastatic CRC. Data from 5 clinical trials and 149 patients supported the FDA label. In addition to patients from the KEYNOTE-016 (NCT01876511, N = 58), data were used from KEYNOTE-164 (NCT02460198, N = 61) (Le et al., 2016), KEYNOTE-012 (NCT01848834, N = 6) (Seiwert et al., 2016), KEYNOTE-028 (NCT02054806, N = 5), and KEYNOTE-158 (NCT02628067, N = 19) trials (Schellens et al., 2017). Ninety patients had CRC and 59 had other non-CRCs, including EC, biliary cancer, GC, pancreatic cancer, small intestinal cancer, breast cancer, prostate cancer, bladder cancer, esophageal cancer, sarcoma, thyroid cancer, retroperitoneal adenocarcinoma, small cell lung cancer, and renal cell cancer. Taken together, the efficacy analysis showed an ORR of 39.6% (95% CI: 31.7, 47.9) complete response (CR) rate of 7.4% (N = 11) and a partial response (PR) rate of 32.2% (N = 48). Specifically, the ORR was 36% in CRC patients (95% CI: 26%, 46%) and 46% in non–CRC patients. (95% CI: 33%, 59%). At the time of data cutoff, median duration of response had not yet been reached (range 1.6+ to 22.7+ months). Among responders, 78% had responses of 6 months or greater. These data led to the presumption that MSI-H phenotype can be a surrogate, pan-cancer, genetic biomarker of distinct subsets of tumors which predict for response to immunotherapy, regardless of tumor histology. Importantly, this is the first histology agnostic FDA approval.

The follow-up data about the clinical trial KEYNOTE-016 were recently reported (ClinicalTrials.gov number NCT01876511) (Le et al., 2017). In a total of eighty patients treated, with a median follow-up time of 12.5 months, we observed ORR in 53% (52% in CRC and 54% in non-CRC) [95% CI, 42 to 64%], with 21% achieving a CR. Neither median PFS nor median OS were reached.

Subsequently, Overman and colleagues provided further evidence for the use of PD-1 inhibitors in chemo-refractory, dMMR/MSI-H mCRC by reporting the outcome of patients treated with nivolumab, a fully human immunoglobulin G4 monoclonal antibody inhibitor of PD-1, in the multicenter, CheckMate 142 phase 2 trial. Patients received nivolumab 3 mg/kg every 14 days or nivolumab plus ipilimumab (anti-CTLA-4) 1 mg/kg every 21 days for four doses followed by nivolumab 3 mg/kg every 14 days until progression (Overman et al., 2017). In the monotherapy cohort, the ORR was 31.3% (95% CI 20.8–42·9), thus achieving the primary endpoint of the study. Nivolumab provided sustained disease control (disease control rate ≥ 12 weeks, 69%), PFS rates of 54% and 50% (at 9 and 12 months respectively), and OS rates of 78% and 73% at 9 months and 12 months. Based on these results, nivolumab was approved in the United States for the treatment of adult and pediatric (age ≥ 12 years) patients with dMMR/MSI-H mCRC who progressed after treatment with a fluoropyrimidines, oxaliplatin, and irinotecan. Longer-term follow up of the CheckMate-142 trial, as presented at the 2018 Gastrointestinal Cancers Symposium, continues to support the use of nivolumab monotherapy in previously treated dMMR/MSI-H CRC (Overman et al., 2018). At a median follow-up of 21 months, the ORR was 34%, with 9% CRs. The median PFS was 6.6 months and the median OS was not reached.

These data formed the nidus for the hypothesis that nivolumab would also have activity in dMMR non-CRC. The NCI's landmark Molecular Analysis for Therapy Choice (NCI-MATCH) launched a trial of nivolumab in patients with dMMR solid tumor. Preliminary results from the first 35 patients treated and followed for at least 6 months were presented during the 2018 Annual Meeting of the Society of Immunotherapy for Cancer. The most common histologies among these patients were endometrioid EC (10 patients), prostate cancer (6 patients), and breast cancer (3 patients). MMR deficiency was defined through IHC as loss of nuclear expression of MLH1 or MSH2. CRC patients were excluded. The study met its primary endpoint: the confirmed ORR rate was 24%, and an additional 27% of patients had stable disease. The 6-month PFS was 49% (Azad et al., 2017).

The combination of nivolumab plus ipilimumab, a fully human immunoglobulin G1 monoclonal antibody that targets CTLA-4 checkpoint receptor, has shown to be synergistic both in preclinical and clinical settings, as the combination is approved for the treatment of metastatic melanoma and is under investigation in the aforementioned phase 2, non-randomized, CheckMate-142, in dMMR/MSI-H mCRC.

The preliminary results of the trial for the combination cohort were recently reported after a median duration of follow-up 13.4 month, demonstrating a manageable safety profile and very interesting clinical activity. At the data cutoff, 119 patients with dMMR/MSI-H mCRC were treated and 63% of patients were still receiving treatment. An objective response was achieved in 54.6% (95% CI, 45.2 to 63.8) per investigator assessment, including 3.4% CR. Responses were durable, with 83% of responders lasting ≥6 months. Disease control > 12 weeks was achieved in 80% (95% CI, 71.5 to 86.6) of patients. Median PFS and OS were not reached, while 12-month PFS rates was 71% (95% CI, 61.4 to 78.7) and

Table 3
Examples of correlation between TMB evaluated by WES and MMR status in solid tumors.

Tumor type	Hypermutation definition based on mutation rate	Comments about MSI status	Ref.
Colon cancer	>12 per Mb (median number of total mutations 728)	75% of the hypermutated tumors were MSI	Muzny et al. (2012)
Gastric cancer	>11.4 mutations per Mb	None of the MSS tumors presented a hypermutated phenotype	Bass et al. (2014)
Endometrial cancer	18×10^{-6} mutations per Mb	The MSI endometrioid tumors had a mutation frequency almost tenfold greater than MSS ones	Kandoth et al. (2013)
Biliary	>11.13 per Mb (median number of non-silent mutations 641)	36% hypermutated tumors harbored a mutation in MMR components	Nakamura et al. (2015)
Glioblastoma	>100 exonic mutations	All tumors were bMMRD and were hypermutated	Bouffet et al. (2016)
Glioma	>20 mutations per 1.4 Mb	54.5% of hypermutated tumors exhibited at least one mutated MMR gene.	Hodges et al. (2017)
Melanoma	>100 mutations per tumor	No association with MMR reported	Snyder et al. (2014)
Lung cancer	≥179 mutations per tumor	MSH2 mutation found in 1 of the 14 patients who had hypermutated tumor and durable clinical benefit to pembrolizumab	Collisson et al. (2014)

bMMRD, biallelic mismatch repair deficiency; Mb, megabase; MMR, mismatch repair: MSI, microsatellite instability; Ref., reference; TMB, tumor mutation burden; WES, whole exome sequencing.

12-month OS rate was 85% (95% CI, 77.0 to 90.2), respectively (Overman et al., 2018).

The important caveat is that CheckMate-142 is a non-randomized phase 2 clinical trial, thus non-intended for comparison of monotherapy to combination therapy. Further randomized studies are warranted to identify potential subgroup of patients more likely to benefit from the combination therapy, as well as it would be important to clarify if the long-term effect of the combination therapy is superior to the monotherapy.

Moreover, evaluation of anti-PD-1 in first line setting is also ongoing (KEYNOTE 177, NCT02563002).

This growing body of research has paved a path for ICI as novel strategies across a range of solid tumors with MMR deficiency, independent of their histologies, but exclusively dependent on a strong relationship between cancer genetics, immune system and therapeutics and several clinical trials are underway (Table 3). We strongly believe that MMR/MSI testing can no longer be considered solely as a screening test to for genetic susceptibility or treatment selection in early-stage CRC but should now be routinely offered to a majority of patients with metastatic cancers, regardless of the site and the histology.

9. MMR deficiency, mutational burden and checkpoint inhibitors in pediatric cancers

The excitement around immunotherapy as a means for durable disease response for dMMR cancer treatment has extended to the pediatric population as well (Majzner, Heitzeneder, & Mackall, 2017). However, efficacy of anti PD-1 antibodies, and their approval for pediatric patients with MSI-H cancers, were extrapolated from the results in the adult MSI-H population (Davis, Agarwal, & Verma, 2017; van Dam, de Zwart, & Meyer-Wentrup, 2015).

Biallelic mismatch repair deficiency (bMMRD syndrome) results from germline-inactivating mutations in DNA MMR genes (Majzner et al., 2017; Tabori et al., 2017). The mode of inheritance is consistent with an autosomal recessive pattern, caused by biallelic mutations that can involve any of the MMR genes (MSH2, MLH1, MSH6, and PMS2). The most commonly involved genes are PMS2 and MSH6, whereas MSH2 and MLH1 mutations are rare, in contrast to the genetic pattern most commonly seen in LS (Bakry et al., 2014). The hallmark of the disease is its high penetrance rate, and very early onset (median age 7.5 years) (Bodo et al., 2015; Lavoine et al., 2015). The spectrum of tumors observed in bMMRD is distinct from LS-related one: the most common malignancies are malignant brain tumors, followed by gastrointestinal and hematologic malignancies. Loss of the MMR protein in both malignant and normal cells assessed by IHC is highly suggestive for a diagnosis of bMMRD and guides subsequent confirmation by mutation analysis in the four MMR genes. Importantly, contrary to LS tumors diagnosis, MSI is not a sensitive nor specific tool for bMMRD diagnosis, especially in non-gastrointestinal cancers (Bodo et al., 2015).

Regardless of the origin (sporadic, LS-related, or bMMRD-related) pediatric dMMR tumors, share a common tumor phenotype and therapeutic challenges, since several chemotherapeutic agents, frequently used in pediatric malignancies treatment, require adequate MMR to exert their tumor damage, such as mercaptopurine and temozolomide (Karran & Attard, 2008). On the other hand, exciting emerging data of immune-based therapies may result in uniquely designed protocols for the management of dMMR cancers, also in the pediatric setting.

A case report of nivolumab administration to two siblings, pediatric patients with GBM with hereditary bMMRD demonstrate impressive antitumor effects with complete radiological resolution of tumors in both patients. No severe treatment-related side effects were observed, except for transient seizures early in the treatment course, possibly GBM-related (Bouffet et al., 2016). Moreover, they investigated the mutational landscape in this rare syndrome and reported that bMMRD pediatric GBM have a significantly higher mutational load than sporadic pediatric and all other brain tumors ($P < 0.001$). They showed that this is related to an impressive high mean neoantigen loads, which is seven to 16 times higher than those in melanomas, lung cancers, or MSI-H gastrointestinal cancers ($P < 0.001$) (Bouffet et al., 2016; Shlien et al., 2015). These results may have implications for treatment of dMMR pediatric cancers. Viana-Pereira et al. have reported that 19% of high-grade glioma samples analyzed were MSI-H, significantly higher than that seen with adult tumors (Viana-Pereira et al., 2011). While some of those patients may have had the rare congenital bMMRD syndrome, dMMR may also arise independently outside of this defined cancer predisposition. The role of ICIs is now being actively tested in children with bMMRD (NCT02992964). However, the safety and effectiveness of ICIs in pediatric patients with MSI-H cancers have not been established and up to date there has been less experience with these approaches in this population. Future research is needed to better understand the clinical effects, the benefits and the potential short and long-term side effects of these therapies in the pediatric setting.

10. Conclusions and future perspectives

In this review we demonstrated that the evaluation of DNA MMR in solid tumors and the identification of dMMR has increasing clinical applications, serving as prognostic and predictive biomarkers for chemotherapy and immune-based therapies, while remaining the cornerstone for the diagnosis of LS.

From a diagnostic point of view, the current method of detecting MSI/MMR deficiency have been optimized for CRCs, and whether these techniques can be applied in all tumor types, is still not fully understood. Upon the recent first FDA tissue/site agnostic drug approval of pembrolizumab for advanced, metastatic MSI-H tumors, examining microsatellites in multiple gene sequencing routinely included in NGS panels could become a screening method for MSI.

We showed that dMMR/MSI-H cancers are biologically marked by genomic instability, a high mutation burden and the potential for high

numbers of neoantigens, which can be observed histologically by a high numbers of TILs compared to pMMR cancers. These features predict response to immunotherapy. However, even among dMMR patients, there are non-responders to immune therapy, arising the urgent need to develop more robust predictive biomarkers (e.g. TMB) to be evaluated together. More importantly, MSI accounts only for the minority of cancers, thus extending the benefit of immunotherapy to a wider, MSS, population is the next and more difficult challenge.

Nevertheless, the reciprocal interplay between cancer genetics and cancer immunology is likely far more pervasive then what we know so far, and investigations in this field will reveal new insights into the mechanisms of how tumor intrinsic molecular alterations influence the TME.

Despite all these challenges, our increasing understanding of these complex interactions will open new innovative diagnostic and therapeutic avenues.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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