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# Quantitative analysis of MMR deficiency in dMMR/MSI high CRC and levels of instability: Implications for ICI therapy of dMMR tumors.

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**Background:** Mismatch repair deficiency (dMMR) is observed in ~15% of newly diagnosed colorectal cancer (CRC) tumors. It is routinely scored as a qualitative trait (positive or negative) and, if present, is considered a positive predictive marker for immune checkpoint inhibitors (ICI) therapy not only in patients with metastatic CRC (mCRC), but also in patients with other dMMR solid tumors. However, a significant proportion of these patients experience failure of ICI treatment and no predictive biomarkers exist that can identify such patients. **Methods:** A total of 42 patients with dMMR tumors were selected for analysis, of which 26 had a hereditary Lynch Syndrome (LS) with known pathogenic variants in the MMR genes (MLH1, MSH2, MSH6, PMS2) and 16 had sporadic dMMR CRC due to epigenetic silencing of the MLH1 gene. All tumors were also analyzed for the presence of the RAS/RAF pathway mutations. MMR deficiency was initially evaluated by two standard methods: immunohistochemistry (IHC) and by microsatellite instability (MSI)-PCR on DNA isolated from FFPE tissues with $>$ 50% tumor content. Subsequently, whole exome sequencing (WES) with a mean depth of $>$150 reads was performed on the same DNA samples using the NovaSeq 6000 platform. NGS data were analyzed using the MSI sensor package which evaluates $>$2000 sites with mono- to pentanucleotide repeats. Each site was scored as NGS-MSI unstable if it contained at least 20% unstable reads. The percent of unstable loci was referred to as NGS-MSI level. All tumors with $>$3.5% of unstable loci were classified as NGS-MSI positive. **Results:** A considerable variability in the NGS-MSI levels ranging from 1.5 – 76% was observed in patients that were initially classified as dMMR/PCR-MSI high. CRC tumors of LS patients with deleterious MMR gene mutations and the majority of patients with sporadic CRC due to MLH1 gene promotor methylation had $>$50% unstable loci. In contrast, more than 1/3 of patients with isolated loss of MSH6 and PMS2 detected by IHC had substantially lower NGS-MSI levels of 1.5 to 12%. These patients had a negative PCR-MSI result and their molecular defects were primarily missense mutations in MSH6 and PMS2 genes. It is interesting to note that a low NGS-MSI score ($<$10%) was also detected in patients with sporadic CRC that had MLH1 gene promotor methylation but also the BRAF V600E mutation. **Conclusions:** Our results indicate that dMMR/MSI-H tumors exhibit a high variability in the percentage of unstable loci that correlates with particular molecular backgrounds. A larger study on different types of tumors is warranted, especially in the context of the efficacy of ICI therapy in patients with MMR tumors classified by qualitative MMR/MSI assays. Research Sponsor: Center for Biomolecular Pharmaceutical Analysis, Faculty of Pharmacy, University "Ss. Cyril and Methodius" in Skopje, RN Macedonia; Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov", Macedonian Academy of Sciences and Arts, Skopje, RN Macedonia.