ORIGINAL ARTICLE

Correlation between density of CD8+ T cell infiltrates in microsatellite unstable colorectal cancers and frameshift mutations: a rationale for personalized immunotherapy

Running title: CD8+ cell density correlates with mutations in MSI-CRCs

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Abstract

Colorectal cancers with microsatellite instability (MSI-CRCs) represent 15% of all CRC, including Lynch syndrome as the most frequent hereditary form of this disease. Notably, MSI-CRCs have a higher density of tumor-infiltrating lymphocytes (TILs) than other CRC. This feature is thought to reflect the accumulation of frameshift mutations in sequences that are repeated within gene coding regions, thereby leading to the synthesis of neoantigens recognized by CD8⁺ T cells. However, there has yet to be a clear link established between CD8⁺ TIL density and frameshift mutations in CRC. In this study, we examined this link in 103 MSI-CRC from two independent tumor cohorts where frameshift mutations in 19 genes were analyzed and CD3⁺, CD8⁺ and FOXP3⁺ TIL densities were quantitated. We found that CD8⁺ TIL density correlated positively with the total number of frameshift mutations. TIL densities increased when frameshift mutations were present within the ASTE1, HNF1A or TCF7L2 genes, increasing even further when at least one of these frameshift mutations was present in all tumor cells. Through in vitro assays using engineered antigen-presenting cells, we were able to stimulate peripheral cytotoxic T cells obtained from CRC patients with peptides derived from frameshift mutations found in their tumors. Taken together, our results highlight the importance of a CD8⁺ T cell immune response against MSI-CRC-specific neoantigens, establishing a preclinical rationale to target them as a personalized cellular immunotherapy strategy, an especially appealing goal for patients with Lynch syndrome.

Keywords: Lynch syndrome; Microsatellite instability; Neo-antigen; Cytotoxic T lymphocyte; Frameshift mutation-derived peptide; Tumor infiltrating lymphocyte
Introduction

Colorectal cancers (CRCs) with high density of tumor infiltrating lymphocytes (TILs), especially of CD8$^+$ T lymphocytes (TLs), are associated with a better prognosis (1-4), suggesting that a cytotoxic anti-tumor immune response could control CRC progression. Cytotoxic immune response is part of a complex immune reaction that includes different cell types, one of them being regulatory T cells (Tregs). Tregs are commonly characterized by the expression of the transcription factor FOXP3 and can suppress cytotoxic TL (CTL) activities. CRCs with microsatellite instability (MSI) represent around 15% of CRCs, including Lynch syndrome, the most frequent hereditary form of CRC. MSI-CRCs are due to a defect of the DNA mismatch repair (MMR) system, leading to accumulation of mutations within DNA repeat sequences. Overall, MSI-CRCs are known to have a better prognosis (5-8) and a more dense infiltration of intraepithelial activated CD8$^+$ TLs than microsatellite stable (MSS) CRCs (9-13), suggesting that MSI-CRCs are particularly prone to a local cytotoxic cellular immune response. The link between stronger immunogenicity of MSI-CRCs and MMR deficiency is commonly explained by the accumulation of frameshift mutations within coding sequences and the synthesis of neo-antigens (14). Degradation of such neo-antigens can release immunogenic neo-peptides, presented by Human Leukocyte antigen class I (HLA-I) molecules, on the tumor cell surface, and targeted by a specific CD8$^+$ cellular immune response. In vitro, TLs from MSI-CRC patients have already been activated against some frameshift mutation-derived peptides (FSPs), underlining that such neo-peptides could indeed be immunogenic (15).

We have previously shown, in 52 MSI-CRCs, that frameshift mutation number and spectrum correlated with total (CD3$^+$) TIL density (16). However, to our knowledge, the direct link
between frameshift mutations in MSI tumor cells and TIL sub-population densities has never been shown.

A total of 103 MSI tumors, from two independent series, were analysed for the correlation between frameshift mutations within 19 selected MSI target genes and CD3\(^+\), CD8\(^+\) and FOXP3\(^+\) TIL densities. We found that CD8\(^+\) TIL density correlated with the number and spectrum of frameshift mutations. Furthermore, we provided evidence that specific CD8 cytotoxic anti-tumor T cell responses could be mounted \textit{in vitro} against frameshift mutations present in patients’ tumors, paving the way for developing new personalized immunotherapy strategies.
Material and methods

Tumor samples

For the main series, colorectal tissues were collected from 106 MSI-CRC surgical specimens, at Rouen University Hospital between 2003 and 2009. This series included 26 Lynch patients (7 with $MLH1$ mutation, 15 with $MSH2$ mutation, 3 with $MSH6$ mutation and 1 without MMR gene mutation detected). In agreement with French regulation, all patients gave a written consent for the MMR gene germline mutation analyses.

For each patient, genomic DNA was extracted from paired tumor and normal colorectal tissues as previously described (16).

For the validation series, colorectal tissues and genomic DNA were collected from 35 MSI-CRC patients who underwent a primary resection of their tumor at the Laennec-HEGP Hospitals, Paris, between 1996 and 2004, as previously described (1).

Ethical, legal and social implications of the study were approved by ethical review boards. All experiments were performed according to the Helsinki guidelines.

Microsatellite instability assessment and determination of tumor cell proportion within the tumor samples

MSI was defined as an instability affecting at least two, among the five, consensus mononucleotide repeats ($BAT25$, $BAT26$, $NR21$, $NR22$ and $NR24$) (17) within tumor DNA, compared to normal colon DNA. PCR products were separated on an ABI Prism 3100 DNA sequencer® (Applied Biosystems).

As previously described (16), to evaluate the proportion of tumor cells within each tumor sample, the profile corresponding to the most unstable microsatellite was selected. The
The proportion of tumor cells within a tumor sample was given by the area of the unstable part of this microsatellite profile, divided by the total area of this microsatellite profile (Figure 1A).

**Detection of frameshift mutations in target genes: fluorescent multiplex PCRs**

As previously described (16), two fluorescent multiplex PCRs were performed on paired normal and tumor DNA samples. In brief, 22 short coding repeat in 19 genes were simultaneously PCR-amplified and separated on an ABI Prism 3100 DNA sequencer®. For each patient, the patterns generated from tumor and paired nonmalignant colorectal tissue DNAs were superimposed. Shifts in the lengths of PCR products corresponded to nucleotidic deletions or insertions (*i.e.* frameshift mutations).

We evaluated the proportion of cells harboring each mutation within the tumor sample: for each mutation, on the PCR profile obtained from tumor DNA sample, the area corresponding to mutated DNA was divided by the total area, corresponding to mutated and not mutated DNA. If this percentage was similar to the percentage of malignant cells in the sample, we concluded that this frameshift mutation was present in all tumor cells, given that chromosomal instability is extremely rare in MSI-CRCs (18, 19; Figure 1B). PCRs were performed when both tumor and normal DNA could be obtained (for 87 patients from the main series and the 35 patients from the validation series).

Percentage of mutations within a tumor was given by the number of mutated genes divided by the number of analysed genes.

**Tissue microarray, immunohistochemistry and TIL quantification**

Tissue microarrays (TMAs) were constructed for 86 out of the 106 patients from the main series (for some patients, no tumor tissue was available). Tissue cores of 0.6 mm diameter were inserted in recipient paraffin blocks. Four cores were taken at the tumor center, four at
the invasion margin, and four from distant histologically normal colonic mucosa (Tissue Arrayer®). Four μm-thick sections from these TMA blocks were used for immunohistochemistry staining of CD3+, CD8+ and FOXP3+ cells, as previously described (20), with mouse monoclonal primary anti-CD3, anti-CD8 (Dako) and anti-FOXP3 (AbCam) antibodies. Ventana Bench Mark XT IHC/ISH Staining Module (Ventana Medical Systems) was used according to the manufacturer’s recommended protocol. Each slide, scanned with Mirax Scan® (Zeiss Systems), was analysed as already described (20), with the Chips’N Cheap TMA analysis program, using Aphelion 3.2 software (ADCIS), further optimized for this purpose. On each chip, outside of the mucinous and the lymphoid areas, we manually delineated epithelium and stroma. Percentages of antibody-labeled surfaces were then automatically calculated (Figure 2). For the validation series, different tissue-arraying instrument (Beecher Instruments, Alphelys), anti-CD3 and anti-CD8 monoclonal antibodies (AbCam), and image analysis system (Spot Browser, Alphelys) were used as previously described (1). The density was recorded as the number of positive cells per tissue surface area unit.

**Construction of Artificial Antigen Presenting Cells (AAPCs) expressing FSP30**

As previously described (21), NIH/3T3 fibroblasts were sequentially transduced with five replication-defective gamma-retroviral vectors encoding HLA-A*0201 (A*0201 heavy chain and human β2-microglobulin), and three human co-stimulatory molecules ICAM-1 (CD54), LFA-3 (CD58) and B7.1 (CD80). These AAPCs were then transduced with a dicistronic vector encoding a puromycin resistance element and one of the following frameshift peptides: RLSSCVPVA, GMCVKVSSI and VLRTEGEPL, called FSP02 (22), FSP27 and FSP30 (23). The high affinity of these peptides for the HLA-A*0201 molecule was validated using the SYFPEITHI algorithm (24).
Each peptide coding sequence was cloned downstream of the human CD8α leader sequence, for the peptide to be addressed into the endoplasmic reticulum. AAPCs expressing these peptides were selected with puromycin (Sigma-Aldrich) at 8 μg/ml for one week.

Peripheral TL purification and stimulation of antigen-specific CTLs

Peripheral blood mononuclear cells (PBMCs) from HLA-A*02+ CRC patients and healthy donors (HLA phenotype assessed in the laboratory) were used upon informed consent and agreement of the local ethic committee. PBMCs were collected by density centrifugation on a lymphocyte separation medium (Eurobio). The next day, non-activated TLs were negatively sorted using Dynabeads untouched human T cell kit (Invitrogen) according to the manufacturer’s instructions. Irradiated AAPCs (25 Gy) were plated (10⁵ per well) in a 24-well plate the day before as previously described (25). T cells were added (1.10⁶ per well) to the AAPCs and cultured for 21 days. A second stimulation was performed for 14 days: irradiated AAPCs were plated as for the first stimulation and 3x10⁵ T cells were added per well. IL-2 (Proleukin Chiron) was added at 20 IU/ml to the cocultures every second day from the seventh day of coculture.

Cytotoxicity Assays

As previously described (25), standard ⁵¹Cr release assays were performed using HLA-A*0201+ T2 cells (ATCC) loaded with the different peptides (irrelevant, FSP02, FSP27 and FSP30, synthesized by Rouen University Proteomic Platfrom, IRIB, Inserm U982, France), at 10 μM for 1 hour at room temperature, or using HLA-A*0201+ HCT116 and Colo205 CRC-derived cell lines (ATCC) incubated 24 hours with IFN-γ (Imukin) at 200 IU/ml. T2, HCT116 and Colo205 target cells were labeled with ⁵¹Cr (for 1 hour at 37°C). 5x10³ target cells were
used per well in 96-well U-bottomed plates at different effector to target (E:T) ratios and incubated at 37°C, for 4 hours for the T2 cells, and 18 hours for the CRC cell lines.

**Statistical Analyses**

Associations between dichotomous characteristics (e.g. presence or absence of frameshift mutations) and continuous variables (e.g. cell densities) were assessed using Mann-Whitney non parametric test. Correlations between quantitative variables (e.g. TIL densities and number of frameshift mutations) were assessed using Spearman’s rank correlation coefficient. Kruskal-Wallis post-hoc test was used for pairwise comparisons among three groups.

To select the frameshift mutations associated with tumor infiltration, among 19 studied genes, while accounting for multiple testing, the following procedure was used: a mutation was deemed correlated with TIL density if it was associated with an increased infiltration in at least two among four independent tumor compartments (i.e. epithelium in tumor center, epithelium in invasion front, stroma in tumor center, stroma in invasion front) at the 0.05 level using Mann-Whitney test. This way, the probability to find, only by chance, a mutation associated with TIL density was lower than 5%.
Results

Frameshift mutation number is highly variable but associated with age and some histopathological features.

We studied the mutational status of coding repeat sequences within 19 selected genes by performing comparative multiplex PCRs on tumor and normal colon tissues of 122 MSI tumors from two independent series (Figure 1). Most of the frameshift mutations were single nucleotide deletions (>95%) within the studied mononucleotidic repeat sequences. As previously reported (16), ACVR2, TAF1B, ASTE1 and TGFBR2 were the most frequently mutated genes (in more than 75% of MSI-CRCs). Moreover, in 30% or more of the tumors harboring mutations in ACVR2, TGFBR2 and ASTE1 genes, these given genes were mutated in all malignant cells, whereas TAF1B almost never was (1/99; Supplementary Table 1).

Number of frameshift mutations was correlated with age and some histopathological characteristics, notably the VELIPI (Vascular emboli, lymphatic invasion and perinervous invasion) criteria (Supplementary Table 2), and was highly variable from a tumor to another, ranging from 0 to 18 mutation(s) among the 22 analysed repeat sequences. No association was found between mutation number and MMR gene status (data not shown).

The median number of frameshift mutations per tumor was 9 for the main series and 11 for the validation one.

TIL density is highly variable but associated with frameshift mutation number

In 86 tumors from the main series (for some patients, no tumor tissue was available) and 35 tumors for the validation series, CD3+, CD8+ and FOXP3+ TILs were quantified in the tumor center, the invasion front and in non-malignant distant tissue, using TMAs (Figure 2). For the 52 patients included in both our previous study (16) and this one, CD3+ T cell density found
on TMAs was correlated ($P = .0001$) with the ones previously found on representative fields of whole slides, confirming the reliability of this TMA-based analysis (Supplementary Figure 1). For the 35 tumors from the validation series, CD3$^+$, CD8$^+$ and FOXP3$^+$ TILs were quantified in the tumor center and the invasion front, using another TMA-based analysis system (as mentioned in the Material and Methods section), which reliability had also been previously verified on 230 colorectal tumors ($P < .0001$, data not shown).

TIL densities highly differed between tumors, ranging from 0.16% to 16.5% for CD3, 0.06% to 13% for CD8, and 0.03% to 1.18% for FOXP3 in the main series (percentages of antibody-labeled surfaces), and from 44 to 1320 cells/mm$^2$ for CD3, 10 to 1020 cells/mm$^2$ for CD8, and 0 to 127 cells/mm$^2$ for FOXP3 in the validation series.

Looking for correlations between TIL densities in whole tumor tissues and increasing frameshift mutation percentages, we found that, in both series, only CD8$^+$ TIL density significantly increased with the percentage of frameshift mutations (Figure 3 and Supplementary Figure 2A). There was a tendency for CD3$^+$ TIL density to increase with this percentage, whereas FOXP3$^+$ TIL density was not higher in tumors containing more mutations.

**ASTE1, HNF1A and TCF7L2 frameshift mutations are associated with higher CD8$^+$ TIL densities**

In the main series, we looked for unbiased robust associations between frameshift mutations in each of the 19 selected genes and CD3$^+$, CD8$^+$ and FOXP3$^+$ TIL densities. We found that frameshift mutations of ASTE1, HNF1A (also known as TCF1) and TCF7L2 (also known as TCF4) genes were correlated with an increased CD8$^+$ TIL density ($P < .05$ in at least two among four independent compartments, *i.e.* epithelium in tumor center, epithelium in invasion...
front, stroma in tumor center, stroma in invasion front). *ASTE1* frameshift mutation was also correlated with an increased CD3$^+$ TIL density ($P < .05$ in at least two among four independent compartments). On the contrary, these mutations were associated neither with FOXP3$^+$ infiltration in any compartment, nor with CD3$^+$ or CD8$^+$ infiltration in normal tissue compartments (Table 1).

*ACVR2* frameshift mutation was associated with an increased CD3$^+$ TIL density ($P = .02$ for the tumor center epithelium and $P = .03$ for the invasion front stroma). No mutation was associated with an increased FOXP3$^+$ infiltration.

Then we focused on the mutations correlated with CD8$^+$ TIL density. CD8$^+$ infiltration was significantly higher in tumors mutated in at least *ASTE1*, *HNF1A* or *TCF7L2* gene compared to tumors with no mutation in these genes (Figure 4A). Moreover, in all tumor compartments, CD8$^+$ TIL density further increased when at least one of *ASTE1*, *HNF1A* and *TCF7L2* genes was mutated in all tumor cells, many pairwise comparisons being significant (Figure 4B). The same tendency was observed for CD3$^+$ cell infiltration, although with less strong associations, but not for FOXP3$^+$ cell infiltration (data not shown).

In the validation series, only two patients were non-mutated on *ASTE1*, excluding this gene from further statistical analysis. Nevertheless, the tumors were still significantly more infiltrated with CD8$^+$ T cells when at least *HNF1A* or *TCF7L2* was mutated ($P = .049$ in the total tumor tissue), especially at the invasion front (Supplementary Figure 2B) with a more dense infiltration when all tumor cells were mutated (Supplementary Figure 2C).

**Patients’ peripheral CD8$^+$ TLs can be activated against neo-peptides derived from frameshift mutations present in their tumor.**

We then tested whether tumor-specific frameshift mutations could indeed be immunogenic in MSI-CRCs. Therefore, we developed a functional assay based on *in vitro* peripheral specific
CTL activation with Artificial Antigen Presenting Cells (AAPCs) expressing the most frequent HLA class I molecule (A*0201), the main costimulatory molecules, ICAM-1, LFA-3 and B7.1 (21), and tumor-specific frameshift peptides.

The first HLA-A*02\(^+\) MSI-CRC patient (P1) included in this functional study was a 27 year old-Lynch patient. In P1’s tumor, we detected (-1) mutations in coding repeat sequences of TGFBR2, TAF1B and ASTE1 genes (Figure 5A), leading to the putative synthesis of the following neo-peptides of high affinity for HLA*A0201: RLSSCVPVA (FSP02), GMCVKVSSI (FSP27) and VLRTEGEPL (FSP30). Therefore, we constructed A*0201-restricted AAPCs expressing these frameshift peptides (AAPC\(^{A2.1/FSP02}\), AAPC\(^{A2.1/FSP27}\) and AAPC\(^{A2.1/FSP30}\), Figure 5B). After two co-cultures with AAPCs encoding FSP02, FSP27 or FSP30, P1’s peripheral CTLs could specifically lyse T2 cells pulsed with the corresponding peptide (Figure 5C). Moreover, peripheral CTLs stimulated with AAPC\(^{A2.1/FSP02}\) or AAPC\(^{A2.1/FSP30}\) could specifically lyse the HLA-A*0201\(^+\) MSI-CRC cell line HCT116, which harbors the same mutations as P1 in TGFBR2 and ASTE1 genes. On the contrary, peripheral CTLs stimulated with AAPC\(^{A2.1/FSP27}\) did not lyse HCT116 cells which do not harbor the same mutation as the patient in TAF1B gene (Figure 5D).

We performed similar experiments on another MSI HLA-A*02\(^+\) Lynch patient (P2, 39 year old), whose tumor harbored the (-1) TGFBR2 and (-1) TAF1B frameshift mutations, but not the ASTE1 (-1) mutation. After two stimulations, P2’s peripheral CTLs could specifically lyse T2 cells pulsed with FSP02 and FSP27, but not T2 cells pulsed with FSP30. Moreover, peripheral CTLs stimulated with AAPC\(^{A2.1/FSP02}\) could specifically lyse HCT116 cells (Supplementary Figure 3A).

Functional assays were also performed on four HLA-A*02\(^+\) additional donors: an MSI-CRC Lynch patient (P3, 49 year old), an MSS CRC patient (49 year old) and two healthy donors (26 and 47 year old). In the tumors of both CRC patients, we did not detect mutations in
TGFB2, TAF1B and ASTE1 genes. We co-cultured their peripheral TLs with AAPC\textsuperscript{A2.1/FSP02}, AAPC\textsuperscript{A2.1/FSP27}, AAPC\textsuperscript{A2.1/FSP30} and AAPC\textsuperscript{A2.1} encoding M1m (AAPC\textsuperscript{A2.1/M1m}). M1m is a peptide derived from MART-1, a melanocyte auto-antigen. Here, AAPC\textsuperscript{A2.1/M1m} were used to ascertain TL functionality, since they can easily activate anti-M1m TLs \textit{in vitro} (25). After two rounds of stimulation on the corresponding AAPCs, TLs from these four donors were cytotoxic against T2 cells pulsed with M1m, but not against T2 cells presenting FSP02, FSP27 or FSP30 (Supplementary Figure 3B).
Discussion

In this study, we showed that ACVR2, TAF1B, TGFBR2 and ASTE1 genes harbored frameshift mutations in the majority of the MSI colorectal tumors (>75%), confirming results previously obtained on a smaller series (16).

To determine whether frameshift mutations could lead to an increased density of different TL populations in MSI-CRCs, we studied CD3+, CD8+ and FOXP3+ TILs on 103 patients from two independent series, using TMAs. CD4+ TLs represent a major TIL population which includes both helper and regulatory T cells, but, unfortunately, they had to be kept out of the scope of this work, as it is often the case when large series of patients are studied by immunohistochemistry (1, 2, 12, 26). Indeed, as many groups and as previously discussed (20), we could not find any anti-CD4 antibody allowing a staining of good enough quality, exploitable with a digital image analysis software.

TMA reliability having often been questioned, because of the small tissue areas studied in very heterogeneous tumors (27), we first validated our TMA-based techniques by confirming the concordance of TIL density analysis results obtained after both TMA and whole slide staining.

In our main series, we found that total TIL density was significantly higher within the tumors harboring a higher number of frameshift mutations or a mutation in ASTE1 confirming the results previously obtained in 52 of these patients (16). Surprisingly, we did not confirm the association between PTEN (exon7) mutation and an increased CD3+ tumor infiltration, pointing out how cautious we need to be when interpreting results found on different analysed tumor areas, with different techniques.
Treg density did not change within tumors harboring a high number of frameshift mutations or within tumors harboring particular mutations. Noteworthy, *TGFBR2* frameshift mutation was not associated with FOXP3+ (neither CD3+ nor CD8+) TIL density. This finding does not support the notion that the increased concentration of TGFβ in the tumor microenvironment, due to *TGRβR2* mutation, could lead to the differentiation of effector TILs into regulatory FOXP3+ TILs (28) and to a general increase in TIL density (29,30). The fact that FOXP3+ TIL density was not correlated with frameshift mutations could be due to a lack of FSP-specific infiltrating Tregs able to inhibit FSP-specific CD8+ cytotoxic response, as suggested by Bauer et al. (31).

The most important result of this study was certainly the correlation found between CD8+ TIL density and frameshift mutation number, in MSI-CRC. Proteins derived from these mutations could be degraded into immunogenic peptides responsible for the increased CD8+ TIL density. In line with this, *in vitro* CD8+ T responses against FSPs have already been reported in MSI-CRC patients, although no correlation could be found between these responses and the frameshift mutations present in the tumors (15, 32).

We observed that frameshift mutations in *ASTE1*, *HNF1A* and *TCF7L2* genes were robustly associated with an increased CD8+ TIL density. Mutated *TCF7L2* mRNA expression, in MSI-CRCs, had already been found correlated with a stronger peritumoral lymphoid reaction (33) and with CD3+ infiltration (34), but we showed for the first time, to our knowledge, a correlation of these three mutated genes with an increased CD8+ tumoral infiltration. Moreover, CD8+ TIL densities were higher in tumors harboring *ASTE1*, *HNF1A* or *TCF7L2* mutation in all tumor cells. These correlations suggest that frameshift mutations in these genes can lead to the production of neo-antigens fragmented into particularly immunogenic neo-peptides, recognized by specific CD8+ TILs.
It could be argued that some FSPs could not be presented because frameshift mutations generally give rise to premature termination codon-containing mRNAs which are prone to degradation by Nonsense-Mediated mRNA Decay (NMD, 35). Among the three mutations we found to be robustly correlated with CD8$^+$ infiltration, $ASTE1$ and $TCF7L2$ mRNAs are not predicted to be degraded by NMD but $HNF1A$ mRNA is. However NMD is not a completely efficient mechanism in tumor cells (36, 37) and, moreover, it has been shown that a major source of antigenic peptides for the MHC-I pathway is the pioneer round of mRNA translation that precedes putative NMD (38).

CD8$^+$ TIL density was not correlated with prognosis in these independent series of MSI-CRC patients (20). Our series might be too small for robust survival statistical analysis, but there could also be a balance between beneficial effects of the immune response and deleterious effects of some studied mutations, many of the studied genes being tumor suppressor genes. Moreover, the correlations found between CD8$^+$ cell density and frameshift mutation number and spectrum suggest that specific CD8$^+$ TLs could be retained at the tumor site, but more detailed TIL in situ function studies, especially of activity markers such as granzymes, perforines, or cytokines, would be needed to better understand the relationships between these mutations and the immune cells.

After specific activation with AAPCs, MSI-CRC patients' peripheral TLs could recognize FSPs derived from frameshift mutations present in their tumor, especially the most correlated one with CD8$^+$ TIL density, i.e. $ASTE1$ (-1) mutation, and the most studied one in terms of immunogenicity, i.e. $TGFBR2$ (-1) mutation (22). On the contrary, peripheral TLs from CRC patients whose tumors did not harbor these mutations or from healthy donors could not be activated with AAPCs encoding the same FSPs. This strongly suggests that, in vivo, the patient’s TLs had already encountered these FSPs, expressed by the patient’s tumor cells, allowing an in vitro memory specific recall response against the same antigen.
Altogether, this work establishes the link between frameshift mutations and CD8$^+$ TL tumor infiltration in MSI-CRC patients, and emphasizes the interest, in MSI-CRC patients and especially in young Lynch syndrome patients, of developing personalized cellular adoptive immunotherapy strategies based on *in vitro* stimulation of their own CTLs against tumor-specific immunogenic neo-peptides derived from frameshift mutations found in their tumor.
References


Table 1. Associations between somatic frameshift mutations and CD3⁺, CD8⁺ and FOXP3⁺ TIL densities.

P values are given for the frameshift mutations in ASTE1, HNF1A and TCF7L2 genes, that were deemed robustly correlated with CD8⁺ TL density, i.e. significantly associated with an increased CD8⁺ density in at least two tumor compartments among the four following ones: tumor center (TC) epithelium (E) and stroma (S), and invasion front (IF) E and S (Mann-Whitney tests). ns, not significant; grey squares, cell density significantly higher when the given gene is mutated.

Figure 1. Fluorescent multiplex PCR frameshift mutation analysis.

(A) MSI status was determined with five consensus mononucleotide repeats (BAT25, BAT26, NR21, NR22 and NR24). For this tumor, all five markers were unstable. To evaluate the percentage of tumor cells within the tumor sample, on the most unstable microsatellite, the area corresponding to mutated DNA was divided by the total area of this microsatellite tumor profile.

(B) Frameshift mutations were characterized based on multiplex PCR profiles. To detect frameshift mutations, mononucleotide repeats of 19 genes were simultaneously amplified in 2 multiplex PCRs. Tumor and non-tumor DNA sample profiles were superimposed and shifts between profiles were identified. For this tumor, one of the two multiplex PCRs revealed that ACVR2, IGFR2, SEC63, TGFBR2, OGT, MSH6 and TCF7L2 were mutated. To evaluate the percentage of cells harboring a given mutation (here a (-1) mutation in ACVR2) within a tumor sample, the area corresponding to mutated DNA was divided by the total area of this
gene tumor profile. The percentage of cells harboring the given mutation divided by the percentage of tumor cells in the tumor sample gives the proportion of tumor cells harboring this mutation.

**Figure 2.** CD3⁺, CD8⁺ and FOXP3⁺ tumor-infiltrating lymphocytes in the tumor center, the invasion front and an associated normal tissue.

For each region, 4 cores (0.6 mm of diameter) were stained with anti-CD3, anti-CD8 or anti-FOXP3 antibody and revealed by peroxidase reaction. The regions of interest (stroma in red and epithelium in blue) and the excluded areas (in black) were manually delineated. Immunostained areas were automatically calculated and circled in yellow. For each region, representative cores from the same tissue sample are shown.

**Figure 3.** Associations between frameshift mutation percentages and CD3⁺, CD8⁺ and FOXP3⁺ tumor-infiltrating lymphocyte densities.

For 68 patients, X axis gives the percentage of mutations found within the 22 studied coding repeat sequences and Y axis gives the percentage of total stained areas calculated from tissue micro array (TMA) immunostained cores (Spearman tests).

**Figure 4.** Associations between ASTE1, HNF1A and TCF7L2 mutations and CD8⁺ TIL densities.

(A) CD8⁺ TIL density in tumors harboring no frameshift mutation or at least one frameshift mutation in ASTE1, HNF1A or TCF7L2 gene. Mean values are shown with SEM. Mann-Whitney tests were performed. (B) CD8⁺ TIL density in the tumors harboring no mutation, at least one mutation in some tumor cells only and at least one mutation within all tumor cells, in ASTE1, HNF1A or TCF7L2 gene. Bars indicate mean values and error bars, SEM. Pairwise
comparisons were performed with a Kruskal-Wallis post-hoc test. (A and B) *, $P< .05$; **, $P< .01$ and ***, $P< .001$.

**Figure 5.** *In vitro* activation of anti-tumor frameshift mutation derived peptide (FSP)-specific T cells from an MSI-CRC patient.

(A) Percentage of tumor cells within patient 1 (P1)’s tumor sample was evaluated on the PCR profile of the most unstable MSI marker, NR22. Mutations in target genes were detected in two multiplex PCRs. Three frameshift mutations, potentially leading to the synthesis of FSPs, were present in more than a third of all tumor cells: $\text{TGFBR2} (-1)$, $\text{TAF1B} (-1)$ and $\text{ASTE1} (-1)$.

(B) Construction of Artificial Antigen Presenting Cells expressing FSP02, FSP27 or FSP30 ($\text{AAPC}^{\text{A2.1/FSP02}}$, $\text{AAPC}^{\text{A2.1/FSP27}}$ and $\text{AAPC}^{\text{A2.1/FSP30}}$).

Non replicative dicistronic gammaretroviral vector encoding FSP02, FSP27 or FSP30 and a puromycin resistance element (puromycin-N-acetyltransferase, puroR) in two coding sequences separated by an internal ribosome entry site (IRES) was used. LTR: long terminal repeat, hCD8αL: human CD8α leader, SD: splice donor site, SA: splice acceptor site, Ψ+: extended packaging signal.

(C) Specific cytotoxic activity of P1’s peripheral TLs stimulated with $\text{AAPC}^{\text{A2.1/FSP02}}$, $\text{AAPC}^{\text{A2.1/FSP27}}$ or $\text{AAPC}^{\text{A2.1/FSP30}}$ was assessed in standard 51Cr release assays on HLA-A*0201+ T2 cells pulsed with an irrelevant or a relevant (FSP02, FSP27 or FSP30) peptide, at different effector to target (E:T) ratios.

(D) Upper panel: for both HLA-A*0201+ CRC cell lines, HCT116 (MSI) and Colo205 (MSS), $\text{TGFBR2}$, $\text{TAF1B}$ and $\text{ASTE1}$ multiplex PCR profiles are shown. HCT116 cells harbored $\text{TGFBR2} (-1/-1)$, $\text{TAF1B} (-3/wt)$ and $\text{ASTE1} (-1/-1)$ mutations. Lower panel: specific cytotoxic activity of P1’s peripheral TLs stimulated with $\text{AAPC}^{\text{A2.1/FSP02}}$, $\text{AAPC}^{\text{A2.1/FSP27}}$ or
AAPC^A2.1/FSP30 was assessed in standard ^51Cr release assays on HCT116 and Colo205 cell lines, at different E:T ratios.
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<th>FOXP3</th>
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Microsatellite instability profiles

Percentage of malignant cells = \frac{\text{Mutated DNA peak area (red)}}{\text{Total tumor DNA peaks area (red + blue)}} \times 100 \quad (\text{in this sample} = 80\%)

Multiplex PCR profiles

Percentage of cells = \frac{\text{Mutated DNA peak area (red)}}{\text{Total tumor DNA peak area (red + blue)}} \times 100 \quad (\text{in this sample} = 80\%)

Percentage of malignant cells = \frac{\text{Percentage of cells harboring the mutation}}{\text{Percentage of malignant cells in the tumor sample}} \times 100 \quad (\text{in this sample} = 100\%)
Figure 2

Tumor Center | Invasion Front | Normal Tissue

CD3

CD8

FoxP3
Figure 3

CD3+

CD8+

FOXP3+

Cell density (% of stained areas) vs. % of frameshift mutations

rho = .124
P = .315

rho = .251
P = .039

rho = -.003
P = .659
Figure 4

A

Epithelium (E) | Stroma (S) | E+ S

Invasion Front (IF)

TC + IF

CD8+ cell density (% of stained areas)

No mutation
≥ 1 mutation

B

Epithelium (E) | Stroma (S) | E+ S

Invasion Front (IF)

TC + IF

CD8+ cell densities (% of stained areas)

No mutation
1 mutation
≥ 1 mutation
NR22 (-10) 65% of tumor cells within the tumor sample

A

TGFBR2 (-1) 55% of tumor cells
TAF1B (-1) 45% of tumor cells
ASTE1 (-1) 60% of tumor cells

B

R L S S C V P V A
G M C V K V S S I
V L R T E G E P L

(FSP27)
(FSP30)

C

AAPC^A2.1/FSP02
AAPC^A2.1/FSP27
AAPC^A2.1/FSP30

% of cell lysis

E:T ratio

D

TGFBR2
TAF1B
ASTE1

HCT116 (MSI)
Colo205 (MSS)

HCT116:
TGFBR2 (-1)
TAF1B (-3)
ASTE1 (-1)
Correlation between density of CD8+ T cell infiltrates in microsatellite unstable colorectal cancers and frameshift mutations: a rationale for personalized immunotherapy

Pauline Maby, David Tougeron, Mohamad Hamieh, et al.

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