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Interleukin 6 Alters Localization of hMSH3, Leading to DNA Mismatch Repair Defects in Colorectal Cancer Cells

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Abstract

Background & Aims—Elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) is the most common DNA mismatch repair (MMR) defect in colorectal cancers, observed in ~60% of specimens. This acquired genotype correlates with metastasis and poor outcome of patients, and is associated with intra-epithelial inflammation and heterogeneous nuclear levels of the MMR protein hMSH3. Inflammation and accompanying oxidative stress can cause hMSH3 to change its intracellular location, but little is known about the source of oxidative stress in cancer cells. We investigated whether cytokines mediate this process.

Methods—We analyzed levels of interleukin 6 (IL6) and its receptor (IL6R) in human colon and lung cancer cell lines by flow cytometry and ELISA; proteins were localized by immunofluorescence and immunoblot analyses. IL6 signaling was blocked with antibodies against IL6, soluble sgp130Fc fragments, and the STAT3 inhibitor NSC74859; a constitutively active form of STAT3 was expressed in colon and lung cancer cell lines to replicate IL6R signaling. EMAST was detected by DNA fragment analysis. Immunohistochemistry was used to examine levels of IL6 in 20 colorectal tumor and adjacent non-tumor tissues.

Results—Incubation of colon and lung cancer cell lines with IL6, but not other cytokines, caused hMSH3, but no other MMR proteins, to move from the nucleus to the cytosol after generation of oxidative stress; inhibition of IL6 signaling prevented this shift. Expression of constitutively active STAT3 also caused hMSH3 to translocate from the nucleus to the cytoplasm in cancer cell lines. Incubation of cells with IL6 led to tetranucleotide frameshifts, the signature for EMAST. EMAST-positive colorectal tumors had significantly higher levels of IL6 than EMAST-negative tumors.

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Conclusions—IL6 signaling disrupts the nuclear localization of hMSH3 and DNA repair, leading to EMAST in cancer cell lines. Inflammatory cytokines might therefore promote genetic alterations in human cancer cells.

Keywords

CRC; mutation; signal transduction; signal transducer and activator of transcription 3

INTRODUCTION

Microsatellite instability (MSI), defined by frameshift alterations among mono- and di-nucleotide DNA microsatellite repeats, is an observed feature among ~15% of all colorectal cancers (CRCs) and can be recognized by the loss of DNA mismatch repair (MMR) protein expression (1,2). Two major DNA MMR proteins, hMLH1 and hMSH2, are common heterodimer partners to other “minor” MMR proteins (e.g. hMSH2-hMSH6, hMSH2-hMSH3, hMLH1-hPMS2) that allow MMR function within the nucleus and control of its protein stability, and both of which when absent completely abrogate DNA MMR function (2). Both hMLH1 and hMSH2 are the most common targets for mutation in the germline of Lynch syndrome patients, a group that shares extremely high risk for CRC and other cancers of the female reproductive track, gastrointestinal track, and urological track (2–5). Among patients with sporadic MSI CRC, hypermethylation of *hMLH1* is the etiology for DNA MMR dysfunction (6). Lynch patients and sporadic MSI CRC patients generally demonstrate longer survival from their cancers when compared to same-staged patients whose tumors retain DNA MMR function, and these patients lack clinical response to 5-fluorouracil-based chemotherapy (2,7,8).

Another form of microsatellite alteration, elevated microsatellite alterations at selected tetranucleotide repeats (EMAST), is observed in some ovarian cancers (9), lung cancers (10), and bladder cancers (11), and had not been associated with DNA MMR defects. More recently, EMAST has been observed in 60% of colon cancers (12–14) and 33% of rectal cancers (15), and has been strongly associated with infiltrating mononuclear inflammatory cells (13,15,16). Reduced, heterogeneous expression of hMSH3 by immunohistochemistry was observed in EMAST-positive tumors (12,13), initially suggesting that this DNA MMR protein might be responsible for EMAST formation. Indeed, hMSH3 knockdown experiments utilizing cell models that can measure EMAST generation proved that hMSH3 deficiency drives EMAST formation (17,18). In contrast to classic MSI, patients with EMAST-positive tumors demonstrate shorter survival when compared to patients with EMAST-negative tumors (15,19).

EMAST appears to be an acquired genotype within cancers (there is no description of a germline hMSH3 mutation to date) that may be a result of chronic inflammation. We previously demonstrated that oxidative stress in the form of H₂O₂ (simulating inflammation) induced a reversible, nuclear-to-cytosolic shift for hMSH3 within three hours of exposure, generating a loss-of-function phenotype for this MMR protein (17). Oxidative stress may be generated as a result of cytokine signaling, and several pro-inflammatory cytokines can

generate oxidative stress, including TNF- α , IL-6, and others, resulting in tissue/cell damage (20).

In particular, IL-6 can either signal through a membrane bound receptor (mIL-6R) as part of its classic pathway, or through a trans-signaling pathway involving a soluble IL-6R (sIL-6R) generated through alternative splicing or shedding. Both mIL-6R and sIL-6R then interact with glycoprotein 130 (gp130) on the cell membrane to transduce an intracellular signal via activation of Janus kinase (JAK) followed by activation of Signal Transducers and Activators of Transcription 3 (STAT3). Activation of STAT3 requires phosphorylation of Tyr705 (pSTAT3^{Tyr705}), resulting in its dimerization and translocation into the nucleus, as well as phosphorylation of Ser727 (pSTAT3^{Ser727}), required to maximize its transcription activity (21). The effects of IL-6 type cytokines also include induction of the MAPK cascade, which ultimately leads to activation of the RAS–RAF–MAPK cascade (21). IL-6 studies have suggested that classic IL-6 pathway is mainly involved in normal developmental processes and tissue homeostasis, while trans-signaling pathway plays a role in acute phase immune responses, inflammation diseases, and cancer development (22).

We hypothesized that one or more cytokines would be responsible to generate oxidative stress and induce a change for hMSH3 compartmentalization. We report that IL-6 induces subcellular compartmental shift of hMSH3 in human CRC and lung cancer cells, as well as human colonic epithelial cells immortalized by cdk4 and hTERT (23), indicating the effect of IL-6 in shifting hMSH3 is not restricted by cell type or neoplastic transformation. IL-6 signals through its trans-signaling pathway, activating STAT3 to drive hMSH3 out of the nucleus. With hMSH3 out of its nuclear compartment where it normally participates in DNA MMR, IL-6 treatment caused tetranucleotide frameshift mutations to accumulate, the signature for EMAS, indicating that a rapid but permanent effect for hMSH3's loss-of-function is recorded in affected cells.

MATERIALS AND METHODS

Cell lines and reagents

The human colon cancer cell lines (HT29, SW480, and SW620) and lung cancer cell line (A549) were purchased from American Type Culture Collection (ATCC; Manassas; Virginia). SW480 cells were cultured as described previously (17). HT29, SW620, and A549 were cultured in DMEM supplemented with 10% FBS, 1X Pen-Strep, and 50 μ g/ml Geneticin. Human Colonic Epithelial Cells (HCEC-1CT) cells were kindly provided by Dr. Jerry Shay at University of Texas Southwestern (Dallas, Texas) (23). Cells were maintained in basal X media (DMEM and Medium 199, 4:1; Gibco; Grand Island, NY), supplemented with 20 ng/ml human EGF (Gibco), 1 μ g/ml hydrocortisone (Sigma; St. Louis, MO), 10 μ g/ml Insulin (Gibco), 2 μ g/ml Transferrin (Sigma), 5 nM sodium selenite (Sigma), 2% Cosmic Calf serum (Hyclone; Waltham, MA), 1X Pen-Strep (Gibco), and 50 μ g/ml Geneticin (Gibco). Cells were cultured in DMEM supplemented with 10% FBS, 1X Pen-Strep, and 50 μ g/ml Geneticin at least three days before and during IL-6 experimentation to avoid interference of IL-6 signaling pathway by the supplements in the maintenance culture media for HCEC-1CT cells. Anti-hMLH1, hMSH2, hMSH3, and hMSH6 antibodies were purchased from BD Pharmingen (San Diego, CA). Anti-tubulin antibody was from Sigma.

HRP-conjugated anti-mouse antibody was purchased from Cell Signaling (Danvers, MA). Alexa 488 conjugated anti-mouse antibody and Alexa 589 conjugated anti-rabbit antibody were from Invitrogen.

Immunofluorescence Microscopy (IFM)

Twenty-five thousands cells per well were seeded onto 8-chamber slides (Invitrogen) and incubated at 37°C/5% CO₂ overnight, followed by serum-starvation for 24 hrs before IL-6 treatment (1 ng/ml; R & D System; Minneapolis, MN) for 16–18 hrs at 37°C/5% CO₂. Cells were briefly washed with PBS, fixed in cold acetone on ice for 5 min, air dried, and stored at 4°C until staining. For staining, cells were re-hydrated/blocked in 5% FBS/PBS at RT for 20 min before incubating with mouse anti-hMSH3 antibody (1:50; BD Biosciences; San Jose, CA) and/or rabbit anti-FLAG (1:500; Cell Signaling, Boston, MA) in 1% FBS/PBS at RT for 1–2 hr. After washing 3 times with 1% FBS/PBS (10 min each wash), cells were incubated with Alexa 488 conjugated anti-mouse and/or Alexa 589 conjugated anti-rabbit antibodies (Invitrogen; 1:1000) at RT for 1.5 hr. Cells were washed as described above and mounted with Prolong Gold with DAPI (Invitrogen). Pictures were taken using an Olympus fluorescent microscope.

Whole cell lysate fractionation and Western-Blotting (WB)

Two hundred fifty thousand cells were seeded onto 6-well plates per well and incubated at 37°C/5% CO₂ overnight. After IL-6 treatment (see above), whole cell lysates were prepared, fractionated, and subjected to WB as documented previously (17).

Detection of hMLH1, hMSH2, hMSH3, and hMSH6 by WB was performed as described previously (17). For the WB detection of pSTAT3 and/or STAT3, anti-pSTAT3 (Tyr705; 1:1000; Cell Signaling), anti-pSTAT3 (Try705; 1:1000; Calbiochem, Darmstadt, Germany; only for Supplemental Figure 5), anti-pSTAT3 (Ser727; 1:1000; Cell signaling), and STAT3 (1:2000; Cell Signaling) were used.

Detection of ROS generation

Cells were seeded onto 8-chamber slides and treated with IL-6 as above. At each time point, ROS generation was monitored using Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Invitrogen) following manufacturer's instructions. Briefly, cells were washed gently with HBSS/Ca/Mg (Invitrogen) before incubated with 25 µM Carboxy-H2DCFDA at 37°C for 30 min, protected from light. Cells were gently washed with warm HBSS/Ca/Mg three times and imaged immediately. Pictures were taken using an Olympus fluorescent microscope.

Flow cytometry to detect membrane bound IL-6 receptors (mIL-6R)

Exponentially growing cells were trypsinized, collected, and counted. One million cells in 100 µl staining buffer (Biolegend; San Diego, CA) were incubated with Human Trustain FcX (Biolegend) on ice for 10 min and then PE conjugated-anti-IL-6Rα antibody (Biolegend) or PE conjugated–isotype control on ice for 30 min in dark. After washing three times with staining buffer, cells were re-suspended in 100 µl of PBS for Flow cytometry analysis.

Measurement of IL-6 and soluble IL-6R in culture media by Enzyme- Linked Immunosorbent Assay (ELISA)

Quantikine human IL-6 and sIL-6R ELISA kits were purchased from R & D systems (Minneapolis, MN). One million cells were seeded onto 100 mm dishes and grown for 5 days before the culture media was collected and centrifuged at 2K rpm for 3 min to remove cells. Clear supernatant was collected to perform ELISA following manufacturer's instructions. Briefly, 100 µl of Assay Diluent was added into each well, followed by the addition of 100 µl of standards and/or samples into each well and incubation at RT for 2 hr. After the plate was washed for four times, 200 µl of IL-6 or sIL-6R conjugates was added into each well and incubated at RT for another 2 hr. After four washes, 200 µl of substrate solution was added into each well and incubated in dark for 20 min before 50 µl of stop solution was added into each well. The plate was read at 450 nm with wavelength correction at 540 nm.

Inhibition and activation of the IL-6 signaling pathway

To block the signaling pathway, IL-6 was pre-incubated with 10 µg/ml of anti-IL-6 antibody (BD Biosciences) at 37°C in culture media for 30 min before the media was used to treat cells. Soluble gp130 Fc (sgp130Fc; 10 µg/ml; R & D Systems) was directly added into the IL-6-containing media for the treatment. Cells were pre-treated with NSC74859 (100 µM; Selleckchem) for 1 hr at 37°C before the addition of IL-6 and fresh NSC74859. Cells were then subjected to ICC and/or total lysate fractionation to examine hMSH3 localization.

The construct expressing constitutively activated STAT3 (S3c) tagged with FLAG (S3c-FLAG) was a kind gift from Dr. Beverly Barton (UMDNJ, Newark, NY) (24). Forty-eight hours post-transfection, cells were harvested to prepare cell lysates for WB or subsequent total cell lysate fractionation. A fraction of transfected cells were seeded onto 4-well chamber slides. Cells on the slides were fixed 48-hr post transfection for IFM studies.

Detection of mutation of intrinsic DNA tetranucleotide repeats

Cells were treated with IL-6 as above for 6 weeks (twice a week) in total. Genomic DNA was isolated at 2-, 4-, and/or 6-week time points and matched with untreated cells at the same passage number using Wizard genomic DNA purification kit (Promega, Madison, WI). Microsatellite instability on tetranucleotide repeats was examined on five previously described markers (*MYCL1*, *D8S321*, *D9S242*, *D20S82*, and *D20S85*). Primer sequences are as following:

<i>MYCL1</i>	forward: 5'-6-FAM-TGGCGAGACTCCATCAAAG-3' reverse: 5'-CCTTTTAAGCTGCAACAATTTC-3'
<i>D8S321</i>	forward: 5'-GATGAAAGAATGATAGATTACAG-3' reverse: 5'-6-FAM-ATCTTCTCATGCCATATCTGC-3'
<i>D9S242</i>	forward: 5'-GTGAGAGTTCCTTCTGGC-3' reverse: 5'-6-FAM-ACTCCAGTACAAGACTCTG-3'
<i>D20S82</i>	forward: 5'-GCCTTGATCACACCACTACA-3' reverse: 5'-6-FAM-TGTGGTCACTAAAGTT TCTGCT-3'

D20S85 forward: 5'-GAGTATCCAGAGAGCTATT-3'
reverse: 5'-6-FAM-ATTACAGTGTGAGACCCTG-3'

Each Polymerase Chain Reaction (PCR) contained 50 ng of genomic DNA, 200 µM of dNTPs (each), 1.5 mM MgCl₂, 200 nM of primer (each), and 0.5 U of HotStart Taq DNA polymerase (Qiagen GmbH) in a total volume of 20 µl. The cycling condition was as following: 95°C for 15 min for initial heat activation; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 30 s; and a final extension at 72°C for 10 min. Fluorescence-labeled PCR fragments were analyzed on Applied Biosystems 3730xl DNA Analyzer with the GeneMarker (SoftGenetic LLC, PA). To obtain individual clones for sequencing, the PCR products were subjected to TA-cloning (Promega). Briefly, PCR products were cloned onto the vector via TA cloning separately and transformed into bacteria, then plated onto LB plates containing ampicillin to select for transformants as per the manufacturer's instructions. Ninety-six clones per group were sequenced to determine the frequency of frameshift mutations. The mutation frequency was determined by calculating the percentage of clones that underwent frameshift mutation over the total number of clones sequenced.

Determination of EMAST status of human colorectal cancer tissues

We utilized colorectal cancers from a previously published cohort (8). Cancer and adjacent normal tissue were microdissected to extract DNA, amplified via PCR at six tetranucleotide repeat loci (*MYCL1*, *UT5037*, *D8S321*, *D9S242*, *D20S82*, and *D20S85*), and analyzed to determine any frameshift mutation by DNA fragment analysis. Cancers were EMAST positive if it exhibited instability at two or more markers relative to normal tissue. We compared the first 20 EMAST-positive to the first 20 EMAST-negative samples that we obtained as analysis for EMAST was performed.

Immunohistochemistry (IHC) staining of human colorectal cancers

IHC staining was performed by the Unit for Laboratory Animal Medicine IHC core at University of Michigan. Briefly, the slides were immersed in xylene twice 10 min each and then run through 100%, 95%, 70%, and 50% alcohol for 5 min each. Slides were then rinsed with deionized water before rehydrated in Phosphate Buffered Saline (PBS) for 10 min. Samples were incubated with two drops of 3% hydrogen peroxide for 10 min to quench endogenous peroxide activity. Samples were washed with PBS and then incubated with 5% serum/PBS for 15 min, followed by incubation with primary antibody at 4°C overnight. Samples were washed with PBS three times 5 min each before incubated with secondary antibody for 1 hour. After washing three times with PBS, samples were developed using DAB/AEC chromogen solution (R & D System). Samples were rinsed three times with PBS, rinsed in deionized water, and mounted with mounting media with hematoxylin. Slides were air-dry overnight. Staining was visualized under a microscope using bright-field illumination. The slides used for determining EMAST status and for IL-6 staining were serial sections of the same tumor. IL-6 staining scoring was performed independently and separately from the EMAST status; thus, IL-6 analysis was blinded from the EMAST status of the tissue. IL-6 staining scores were analyzed using Mann-Whitney U-test (IBM SPSS software).

RESULTS

IL-6 treatment induces a nuclear-to-cytosol shift of hMSH3 in cancer cells

Our previous study revealed that oxidative stress via H_2O_2 led to mislocalization of hMSH3 from the nucleus to the cytoplasm (17). We sought to determine the potential source of the oxidative stress that had this effect on hMSH3. Considering the fact that EMAST tumors are tightly associated with inflammation, we adopted immunofluorescence microscopy (IFM) to screen several classic pro-inflammatory cytokines for such effect. Our screening revealed that TNF- α , IL-1 β , IFN- α , and IFN- γ did not exhibit any effects in shifting hMSH3 (Supplemental Fig. 1A). On the contrary, we found IL-6 treatment causes the hMSH3 shift in all cell lines tested, including three human CRC and one lung cancer cell lines (Fig. 1A and Supplemental Fig. 1BCD). These results indicate the effect of IL-6 is not restricted to CRC cells, consistent with the fact that EMAST has been observed in various kinds of cancers, including CRC (12–15) and lung cancer (10), and that CRCs show heterogeneous nuclear hMSH3 expression when EMAST is present (12,13). IL-6 also induced hMSH3 shift in normal human colonic epithelial cells immortalized by cdk4 and hTERT (HCEC1CT; Supplemental Fig. 1E), indicating hMSH3 shift upon IL-6 treatment may be a general phenomenon that does not require any neoplastic or oncogenic alterations. To supplement the IFM data, whole cell lysates were fractionated into nuclear and cytosolic fractions for subsequent Western Blotting (WB) to monitor localization of MMR proteins (Fig. 1B and Supplemental Fig. 2ABCD). IL-6 treatment induces the nuclear-to-cytosol shift of hMSH3 in a dosage-dependent fashion, and this effect is specific to hMSH3 with no change in the localization of hMLH1, hMSH2, and/or hMSH6 (Fig 1B, Supplemental and Supplemental Fig. 2ABCD). IL-6 treatment did not reduce the overall expression levels of hMSH3 in any of the cancer cell lines studied (Supplemental Fig. 3).

Reactive Oxygen Species (ROS) is generated upon IL-6 treatment

To assess if oxidative stress plays a role in hMSH3 shift upon IL-6 treatment, we examined intracellular ROS generation using Live Imagine-iT. ROS generation was readily detected upon IL-6 treatment, accompanied by the hMSH3 shift to the cytoplasm (Fig. 1C). Detailed time-point studies indicate that both ROS generation and hMSH3 shift can be detected as early as 2.5 hours after the treatment, peaks between 8 to 16 hours, and subsides 24 hours after the treatment (Supplemental Fig. 4ABCD). ROS generation correlating with the hMSH3 cytoplasmic shift strongly suggests cause and effect, and builds upon our prior observation of oxidative stress inducing the hMSH3 shift.

Activation of STAT3 is via sIL-6R and not mIL-6R in cancer cells

To delineate the cascade mechanism for the hMSH3 shift upon IL-6 treatment, we employed flow cytometry and ELISA to examine the expression of IL-6, mIL-6R, and sIL-6R. Both SW480 and SW620 colon cancer cells expressed detectable levels of mIL-6R, while HT29 colon cancer and A549 lung cancer cells did not (Fig. 2). Cell culture media was collected to measure the amount of IL-6 and/or sIL-6R secreted into the media by ELISA. Of the four cell lines examined, only A549 cells secreted detectable level of IL-6, and all four cell lines secreted detectable levels of sIL-6R (Table 1). Collectively, these results strongly suggest

that the effect of IL-6 in causing shift of hMSH3 is most likely carried out via IL-6–sIL-6R (trans-signaling) pathway.

Prior studies have firmly established a role of IL-6 in activating STAT3 signaling pathway (21). We investigated if STAT3 activation was involved in the shift of hMSH3 upon IL-6 treatment. To monitor STAT3 activation, cells lysates were prepared at different time points after IL-6 treatment for WB using antibodies specific for STAT3 and phospho-STAT3. Phosphorylation of STAT3 at Tyr705 (pSTAT3^{Tyr705}) only occurred upon IL-6 treatment, except for A549 cells which exhibited basal levels of pSTAT3^{Tyr705} likely due to its native IL-6 secretion (Table 1). In contrast, STAT3^{Ser727} was constitutively phosphorylated at low levels in all four cell lines, but increased phosphorylation levels were detected upon IL-6 treatment (Fig. 3). Replacing HT29 cell culture media with media from SW480 cells (containing higher concentrations of sIL-6R) markedly shortened the time required to yield detectable level of pSTAT3^{Tyr705} in HT29 upon IL-6 treatment (Supplemental Fig. 5). This result further supports the concept that IL-6 trans-signaling is responsible for the observed hMSH3 shift.

IL-6 trans-signaling through sIL-6R and STAT3 activation drives the hMSH3 compartmental shift

To further examine if IL-6–sIL-6R–STAT3 pathway is the cause for the observed hMSH3 shift, we inhibited various portions of the signaling pathway by (a) the addition of an anti-IL-6 antibody to neutralize IL-6, (b) utilizing recombinant sgp130Fc to inhibit the interaction of IL-6–IL-6R with membrane bound gp130, and (c) using the drug NSC74859 to prevent the dimerization and nuclear translocation of phospho-STAT3 (Fig. 4A). Each of these pathway inhibition treatments was able to prevent the hMSH3 compartmental shift (Fig. 4B). Cell lysate fractionation and WB were completely congruent with the IFM results (Fig. 4C). To ensure that the addition of anti-IL-6 antibody and sgp130Fc successfully blocked the pathway, we examined generation of pSTAT3^{Tyr705}. As expected, both treatments were able to markedly reduce the amount of pSTAT3^{Tyr705} (Supplemental Fig. 6A). Additionally, nuclear fractions of proteins from cells treated with NSC738912 were prepared to monitor the nuclear pSTAT3^{Tyr705} levels with and without treatment. Indeed, there was significantly less nuclear pSTAT3^{Tyr705} in cells treated with IL-6 and NSC738912 compared to cells treated with IL-6 alone (Supplemental Fig. 6B), indicating successful blockage of nuclear accumulation of pSTAT3^{Tyr705} by NSC74859. Taken together, these results demonstrate a strong association between the IL-6–sIL-6R–STAT3 pathway and mislocalization of hMSH3. The fact that the addition of sgp130Fc was able to prevent the hMSH3 shift upon IL-6 treatment provides an additional piece of evidence that IL-6 trans-signaling is involved in this process because sgp130Fc only interacts with IL-6–sIL-6R complexes but not IL-6 alone.

To directly test if the activation of STAT3 is capable of causing the shift of hMSH3, an expression vector carrying a constitutively activated mutant STAT3 tagged with FLAG (S3c-FLAG) (24), was transfected into SW480 and/or A549 cells. IFM was employed to examine the localization of S3c-FLAG and hMSH3, hMSH3 (green) was present homogeneously in both nuclear and cytosolic compartments in S3c-FLAG expressing cells

(red) cells, compared to only nuclear hMSH3 in mock transfected cells (Fig. 5A and Supplemental Fig. 7). To supplement the IFM results, total cell lysates were fractionated into nuclear and cytosolic fractions for WB. hMSH3 shuttled to the cytosol in S3c-FLAG transfected cells (Fig. 5B). Our results demonstrate a role of STAT3 in shifting the subcellular localization of hMSH3.

IL-6 treatment causes EMAST in cancer cells

We clearly demonstrate that IL-6 treatment leads to a loss-of-location phenotype for hMSH3 in cancer cells. The question remained if the loss-of-location of hMSH3 was enough to cause loss-of-function and to generate EMAST. We employed fragment analysis to examine five native tetranucleotide EMAST markers (*MYCL1*, *D8S321*, *D9S242*, *D20S82*, and *D20S85*) in cells with and/or without IL-6 treatment. Mutant frameshift peaks were observed for *MYCL1* and *D9S242* in SW620 cells treated with IL-6 (Supplemental Fig. 8AB). Peaks corresponding to frameshift mutation were also detected for *MYCL1* in IL-6-treated HT29 cells (Supplemental Fig. 8C). PCR products corresponding to these markers were sub-cloned, allowing us to sequence individual clones to quantitate mutation frequency. Frameshift mutations were found 8–9 fold higher in IL-6-treated SW620 (Fig. 6A; $P = 0.002818$) and 5-fold higher in HT29 cells (Supplemental Fig. 8D; $P = 0.03$) for *MYCL1*. More detailed sequential mutation analysis conducted at 2, 4, and 6 weeks upon IL-6 treatment revealed that mutation could be detected as early as 2 weeks after treatment ($P = 0.014$). Mutation frequency further increased by approximately 2–3 folds when treatment was prolonged to 4 ($P = 0.0001$) and 6 ($P = 0.0002$) weeks. In sharp contrast, mutation frequency did not change significantly for the control cells (overall $P < 0.0001$; Supplemental Table 1). Altogether, these results confirm IL-6's capability to induce EMAST in human CRC cells.

Higher IL-6 levels correlate with EMAST-positive human CRCs

We immunostained 20 EMAST-positive and 20 EMAST-negative human colorectal tumor samples for IL-6 expression. Thirteen of 20 EMAST-positive exhibited strong IL-6 staining, four with medium staining, and three with weak staining. In contrast, only three EMAST-negative tumors exhibited strong IL-6 staining, four with medium staining, and 13 with weak staining (Fig. 6BC). Using a semi-quantitative score for staining, EMAST-positive tumors significantly expressed higher levels of IL-6 compared with EMAST-negative CRCs ($P=0.001$). This data compliments our cell studies indicating the pro-inflammatory cytokine IL-6 is highly associated with EMAST formation.

DISCUSSION

We previously observed that oxidative stress induced compartmental shift of hMSH3 (17). Here, we sought to determine the source of oxidative stress that can induce this loss-of-location phenotype for hMSH3 by shifting it from the nucleus and sequestering it into the cytoplasm. We discovered that the pro-inflammatory cytokine IL-6 is the signal driver for the hMSH3 compartmental shift. Specifically, we demonstrate that (a) IL-6 signals through its trans-signaling pathway involving sIL-6R to activate STAT3 signaling, (b) IL-6 induces ROS generation, and coincides with hMSH3's shift from the nucleus to the cytosol, (c) IL-6

elicits a dose-dependent shift of hMSH3, (d) inhibition of the IL-6 signaling pathway blocks the hMSH3 shift, and constitutively-activated STAT3 induces hMSH3 shift without IL-6 treatment, and (e) IL-6 treatment increases endogenous tetranucleotide mutation frequency that defines EMAST. IL-6 levels also strongly correlate with EMAST-positive CRCs. Thus, IL-6 drives hMSH3 dysfunction to cause EMAST.

Prior studies have firmly established that STAT3 pathway is one of the major pathways downstream of IL-6-IL-6R (21). Activation of STAT3 can be detected upon IL-6 treatment in all cells we tested. Specifically, pSTAT3^{Tyr705} could only be detected after IL-6 treatment, except for basal levels in A549 cells from intrinsic IL-6 ligand (Fig. 3). On the other hand, pSTAT3^{Ser727} could be detected before treatment with stronger signals observed after treatment. Inhibition at three different steps within the IL-6-sIL-6R-STAT3 pathway prevented the hMSH3 shift (Fig. 4B and 4C). Additionally, transient expression of constitutively activated STAT3 (S3c) was able to induce the hMSH3 shift without IL-6 treatment. Our results revealed that only a fraction of S3c was able to enter into the nucleus, but this was enough to observe higher levels of hMSH3 within the cytoplasm (Fig. 5A and 5B). It is very important to bear in mind that S3c is not phosphorylated at Tyr705 and therefore may not be able to fully exert all of its cellular functions. The S3c construct appears to only modestly shift hMSH3 to the cytoplasm compared to IL-6 treated cells. Nevertheless, a role of STAT3 pathway in shifting hMSH3 is demonstrated with our experiments.

EMAST has been observed in a number of cancers, including small cell cancer of the lung (10), bladder cancer (11), ovarian cancers (9), and colorectal cancers (12–15), and among benign familial hamartomatous polyps in the colon (25). EMAST is strongly associated with inflammation, and its presence is a biomarker for poor prognosis in patients with colorectal cancers (15,16,19). Non-cancer conditions, such as colitis in inflammatory bowel disease and pancreatitis, have been previously assessed for microsatellite instability, and are now known to be MSI-low. One speculation is that MSI-low and EMAST represent the same or a spectrum biomarker of inflammation, as both EMAST and MSI-low often overlap in colon tumors (19,26,27). Given the consequence of metastasis and poor survival with EMAST-positive tumors, we need to understand how EMAST is generated and how it might impact or be modified by therapy. We propose that inflammation in and around tumor epithelial cells, principally driven by IL-6 release from immune and epithelial cells, causes the hMSH3 shift and EMAST, and our experiments support this hypothesis. Indeed, in the present manuscript, we demonstrate that IL-6 induces the hMSH3 shift in normal human colonic epithelial cells immortalized by cdk4 and hTERT. Either reducing the inflammation or directly blocking IL-6 signaling should prevent EMAST formation and its changes to DNA, as well as theoretically improving patient outcome.

How STAT3 activation, downstream from IL-6 signaling, shifts hMSH3 out of the nucleus is not known, and is under intense investigation within our laboratory. Bioinformatics approaches suggest potential nuclear export signals within hMSH3 that are absent or distinct from other DNA MMR proteins, suggesting specificity for hMSH3 shifting. Indeed, hMLH1, hMSH2, and hMSH6 do not shift with IL-6 signaling or oxidative stress. It is unclear why such a mechanism to shift this DNA repair protein out of the nucleus would

evolve. Since there are very few if any genes in the human genome containing tetranucleotide repeats in coding regions that could frameshift with loss of hMSH3 (28), we speculate that frameshifts within non-coding microsatellites areas provide greater genetic diversity for recombination events for normal cells, and provide an avenue for increase metastatic diversity in tumor cells since EMAST is associated with metastasis.

EMAST-positive tumors exhibited stronger IL-6 staining compared to EMAST-negative tumors, indicating a strong association between IL-6 and EMAST formation in tumors from CRC patients. IL-6 from any source would be expected to allow the heterogeneous expression of hMSH3 as described in prior publications (12,13), and allow EMAST formation. We have attempted to stain patient tissues with anti-pSTAT3^{Tyr705} antibody to examine its activation status. Unfortunately, we could not detect any nuclear pSTAT3^{Tyr705} in any tissues we stained. Since phosphorylated proteins are known to be short-lived, freshly cut slides from newer tissue samples may be required for such staining. We intend to revisit this issue in subsequent studies.

In summary, we demonstrate a novel mechanism for DNA MMR dysfunction in cancers with inflammation. The pro-inflammatory cytokine IL-6 signals through its trans-signaling pathway to activate STAT3, which drives hMSH3 from its normal nuclear location to the cytosol, allowing frameshift mutation of DNA to occur at tetranucleotide repeats (EMAST). This hMSH3 “loss-of-function” appears to be the most common type of DNA MMR dysfunction as it is observed in the majority of colorectal cancers, and is reported to be associated with metastatic behavior conferring poor survival. Interference with EMAST formation may therefore positively affect patient outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

MSI	microsatellite instability
EMAST	elevated microsatellite alterations at selected tetranucleotide repeats
CRC	colorectal cancer
MMR	mismatch repair
IDL	insertion/deletion loop
IL-6	Interleukin-6

mIL-6R	membrane-bound IL-6 receptor
sIL-6R	soluble IL-6 receptor
STAT3	Signal Transducers and Activators of Transcription 3

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Fig. 1. IL-6 treatment causes a nuclear-to-cytosol shift for hMSH3

(A) Untreated (control) and/or IL-6-treated SW480 cells were stained with anti-hMSH3 antibody to monitor hMSH3 (green) localization and with DAPI (blue) to localize the nucleus. (B) Total cell extract from untreated and/or treated SW480 cells was fractionated into cytosolic and nuclear fractions, followed by Western blotting to monitor the amount of each protein in cytosolic and nuclear compartments. The numbers provided refer to the relative amount of hMSH3 protein to control proteins, and the table represents the nuclear/cytosolic hMSH3 ratio for each dose of IL-6. (C) ROS generation was readily detected upon IL-6 treatment, accompanied by the subcellular compartmental shift of hMSH3. SW480 cells were treated with IL-6 for 8 hours before cells were used to monitor ROS generation and/or hMSH3 localization.

Fig. 2. SW480 and SW620, but not HT29 and A549, expressed mIL-6R

Cells were stained with anti-IL-6 antibody and/or the isotype control antibody and subjected to flow cytometry analyses to determine mIL-6R expression.

Fig. 3. Phosphorylation (activation) of STAT3 upon IL-6 treatment

Total protein extract was prepared at different time points after the addition of IL-6 for the subsequent Western blots to monitor the phosphorylation of STAT3 at Tyr705 (pSTAT3^{Tyr705}) and/or Ser727 (pSTAT3^{Ser727}). Total STAT3 and α-Tubulin were included as controls.

Fig. 4. IL-6 induces hMSH3 shift from the nucleus to the cytoplasm through IL-6-sIL-6R-STAT3 pathway

(A) Schematic of the IL-6–IL-6R–STAT3 pathway and our strategy to inhibit the pathway.

(B) Cells were stained with anti-hMSH3 antibody (green) and DAPI (blue) to monitor the hMSH3 localization upon various treatments.

(C) Total cell extract was fractionated into cytosolic and nuclear fractions for Western blotting to quantitate the amount of cytosolic and/or nuclear hMSH3. Histone 3 (H3) is used as a nuclear marker and α -Tubulin serves as a cytosolic marker. N/C=nuclear to cytosolic ratio for hMSH3. N/C ratios of zero are fractions smaller than 0.1, and are not truly zero.

Fig. 5. Forced expression of constitutively-activated STAT3 (S3c) led to partial nuclear accumulation of S3c and cytosolic leakage of hMSH3

SW480 and/or A549 cells were transfected with an expression vector carrying constitutively activated STAT3 (S3c) tagged with FLAG (S3c-FLAG). **(A)** Transient transfected cells (top: SW480; bottom: A549) were subjected to immunofluorescence microscopy using anti-FLAG (red), anti-hMSH3 (green), and/or DAPI (blue). hMSH3 (green) is present homogenously in both nuclear and cytosolic compartments in the S3c-FLAG expressing cells (bright red; pointed by white arrows), whereas it is predominately in the nucleus in the neighboring non-transfected cells (pointed by blue arrows). **(B)** Total cell lysates from transient transfected cells were fractionated into nuclear and cytosolic fractions for Western blotting to examine protein localization. Please note the existence of nuclear S3c protein, accompanied by the increase of cytosolic hMSH3, in transfected cells compared to the control cells.

Fig. 6. IL-6 treatment induced EMAST in human CRC cells and EMAST positive tumors exhibited stronger IL-6 staining compared to EMAST-negative tumors

(A) Genomic DNA was isolated from untreated (control) and treated cells. DNA fragments containing different tetranucleotide markers were amplified and sub-cloned onto a vector. Ninety-six clones per group were sequenced to determine the occurrence of frameshift mutations. The percentages of the mutated clones (mutation frequency) for *MYCL1* in untreated and treated SW620 cells are presented. (B) IL-6 staining intensity was classified into four groups: very strong, strong, medium, and weak/negative. Examples are shown here. (C) The staining intensity was assigned a score (very strong = 4; strong = 3; medium = 2; weak = 1; negative = 0), allowing us to evaluate the staining in a semi-quantitative fashion.

Table 1
Levels of IL-6 and sIL-6R in human cancer cells

Cells were cultured for 5 days before culture media was collected to determine IL-6 and/or IL-6R concentration by ELISA. Cells were trypsinized, collected, and counted for the subsequent calculation.

	IL-6 (pg/ml/million cells)	sIL-6R (pg/ml/million cells)
HT29	0	1.06 ± 0.02
SW480	0	52.8 ± 1.29
SW620	0	9 ± 0.62
A549	4.6 ± 0.07	51.4 ± 6.23