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Instability of a dinucleotide repeat in the 3′-untranslated region (UTR) of the microsomal prostaglandin E synthase-1 (*mPGES-1*) gene in microsatellite instability-high (MSI-H) colorectal carcinoma

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ABSTRACT

DNA mismatch-repair gene mutations, with consequent loss of functional protein expression, result in microsatellite instability (MSI). Microsatellite sequences are found in coding regions and in regulatory regions of genes (i.e., 5′-UTRs and 3′-UTRs). In addition to being a surrogate marker of defective mismatch repair, deletion or insertion microsatellite sequences can dysregulate gene expression in MSI-H (microsatellite instability-high) tumors. The microsomal prostaglandin E synthase-1 (*mPGES-1*) gene product, mPGES-1, participates in prostaglandin E_2 (PGE_2) production. Moreover, mPGES-1 is often overexpressed in human colorectal tumors, and is thought to contribute to progression of these tumors. Here we identified a dinucleotide repeat, $(GT)_{24}$, in the *mPGES-1* gene 3′ untranslated region (3′-UTR), and analyzed its mutation frequencies in MSI-H and microsatellite stable (MSS) tumors. The $(GT)_{24}$ repeat exhibited instability in all MSI-H tumors examined (14), but not in any of the MSS tumors (13). In most cases, $(GT)_{24}$ repeat instability resulted in insertion of additional GT units. We also determined *mPGES-1* mRNA levels in MSI-H and MSS colorectal cancer cell lines. Three of four previously designated "MSI-H" cell lines showed higher *mPGES-1* mRNA levels compared to MSS cell lines; correlations between elevated *mPGES-1* mRNA levels and microsatellite $(GT)_{24}$ repeat characteristics are present for all six cell lines. Our results demonstrate that *mPGES-1* is a target gene of defective mismatch repair in human colorectal cancer, with functional consequence.

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1. Introduction

mPGES-1, the protein product of the microsomal prostaglandin E synthase gene (*PTGES*, *mPGES-1*), converts prostaglandin H_2 (the product of both cyclooxygenase 1 and cyclooxygenase 2) to prostaglandin E_2 (PGE_2). Elevated PGE_2 levels are postulated to play a role in colorectal cancer pathogenesis. Increased mPGES-1 protein levels occur in human colorectal adenomas and adenocarcinomas, suggesting *mPGES-1* may play a role in early stages of colon tumor development. (Yoshimatsu et al., 2001; Kamei et al., 2003) *mPGES-1* overexpression in human colorectal cancer (CRC) cell lines leads to increased cell proliferation and increased PGE_2 production, while global *mPGES-1* gene deletion reduced significantly the total number of intestinal polyps in $Apc^{\Delta 14/+}$ mice. (Kamei et al., 2003; Nakanishi et al., 2008) In related studies, *mPGES-1* global deletion reduced the number of aberrant crypt foci, number of polyps, and the size of those tumors that did form in a carcinogen-induced colon cancer mouse model. (Nakanishi et al., 2008, 2011; Sasaki et al., 2012) Taken together, these observations provide strong evidence for a role of *mPGES-1* in colon carcinogenesis.

Mismatch-repair (MMR) deficiency, due to mutation or loss of expression of one or more of the DNA mismatch repair genes, occurs in both familial and sporadic human CRCs. In familial CRCs such as Lynch syndrome, MMR-deficiency is caused by germline inactivating mutations in the *hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2* DNA repair genes; in sporadic CRCs epigenetic modifications such as promoter hypermethylation of the *hMLH1* gene account for the MMR-deficiency. (Marra and Boland, 1995; Herman et al., 1998) As a result of MMR-deficiency, microsatellites (mono, di-, tri-, and tetranucleotide repeats) located in both intergenic regions and in either the coding or regulatory regions of genes may undergo instability, resulting in insertion or deletion of these repeating units. Microsatellite instability (MSI) is seen in ~15% of sporadic CRCs and in most tumors associated with Lynch syndrome. (Marra and Boland, 1995) Instability of microsatellites located in the 5′-untranslated regions (5′-UTR), coding regions, and 3′-untranslated regions (3′-UTR) of genes has been associated with altered gene expression, suggesting MSI represents a mechanism for dysregulating gene function. (Parsons et al., 1995; Baranovskaya et al., 2009; Paun et al., 2009; Yuan et al., 2009; Kim et al., 2013) In this study, we identified a dinucleotide repeat region located in the 3′-UTR of the *mPGES-1* gene and investigated whether the *mPGES-1* gene is a target of defective MMR in human colorectal cancer.

2. Materials and methods

2.1. Microsatellite stable (MSS) and microsatellite instability-high (MSI-H) tumor samples

Twenty-seven de-identified tumor DNA samples, 13 MSS and 14 MSI-H, and their matched normal mucosa were examined in this study. All samples were analyzed previously for their MSI status with the National Cancer Institute (NCI)-recommended reference panel of five microsatellite markers (*BAT25*, *BAT26*, *D2S123*, *D5S346* and *D17S250*) in our clinical laboratory (Boland et al., 1998). Tumor samples exhibiting novel length alleles (i.e., insertion or deletion mutations) at two or more of the five markers were characterized as MSI-H; tumors exhibiting no length changes at all five markers were characterized as MSS (Boland et al., 1998).

2.2. Colorectal cancer cell lines

Four reported MSI-H (HCA7, HCT116, LoVo, and LS174T) and two reported MSS (Caco2 and SW620) CRC lines were used in this study (Di Pietro et al., 2005; Jung et al., 2009; Williams et al., 2010). All cell lines were maintained in Dulbecco's modified eagle's medium (DMEM) at 37 °C and 5% CO_2.

2.3. $(GT)_{24}$ repeat genotyping and microsatellite instability analysis

For tumor samples, DNA was isolated from the microdissected MSS/MSI-H tumors and their matched normal mucosa, using the standard protocols in our clinical laboratory. For CRC lines, DNA was isolated using the Qiagen DNeasy mini kit (Qiagen, Carlsbad, CA). The $(GT)_{24}$ repeat region located in the 3′-UTR of the *mPGES-1* gene was amplified with the following primers: forward primer 5′-GAAACTGCAAATGTCCCCTTGAT-3′ and the reverse primer 5′-CACATCTCAGGTCACGGGTCTA-3′ (6-FAM labeled). The primers are expected to amplify a PCR fragment of 109 bp if there is no expansion or contraction of the repeat (see Figure 1). The PCR amplification conditions used included 35 cycles with a 55 °C melting temperature, and a 30 s extension. Amplified fluorescent PCR products were mixed with formamide and GeneScan™ ROX™ size standard, denatured, and subjected to capillary electrophoresis on an ABI 3130xI Genetic Analyzer. Data were analyzed with GeneMapper Fragment Analysis Software (Applied Biosystems). Each tumor $(GT)_{24}$ repeat microsatellite profile was compared to the profile for its matched normal mucosa. Tumors were characterized as MSI when the tumor sample exhibited a PCR product that demonstrated an elongation and/or contraction when compared to the PCR product from the matched mucosal sample.

2.4. Quantitative real time RT-PCR for mPGES-1 expression

Total RNA was isolated from the six CRC lines, using the Qiagen RNeasy mini kit (Qiagen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using the iSCRIPT cDNA synthesis kit (BioRad, Hercules, CA), using 50 ng of cDNA per reaction as template. Quantitative real time RT-PCR was performed using SYBR green PCR master mix (Applied Biosystems). The *mPGES-1* mRNA levels were normalized to β−actin levels and the relative expression of *mPGES-1* mRNA levels was determined by the comparative CT method (Livak and Schmittgen, 2001).

ccagcagctgatgcctccttggccaccagaccatgggccaagagccgccgtggctatacctggggact
tgatgttccttccagattgtggtgggccctgagtcctggtttcctggcagcctgctgcgcgtgtgggtctctgg
gcacagtgggcctgtgtgtgtgcccgtgtgtgtgtatgtgtgtgtgtatgtttcttagcccctggattcctgca
cgaagtggctgatgggaaccatttcaagacagattgtgaagattgatagaaaatccttcagctaaagta
acagagcatcaaaaacatcactccctctccctccctaacagtgaaaagagagaagggagactctattt
aagattcccaaacctaatgatcatctgaatcccgggctaagaatgcagacttttcagactgaccccaga
aattctggcccagccaatctagaggcaagcctggccatactgtatttttttttttccaagacagagtcttgctc
tgttgcccaagctggagtgaagtggtacaatctggctcactgcagcctccgcctcccgggttcaagcgat
tctcccgcctcagcctcctgagtagctgggattacaggcgcgtatcaccatacccagctaatttttgtatttttt
agtagagacgggttcaccatgttgcccaggagggtctcgaactcctggcctcaagtgatccaccggcct
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gctcccatgtgagtccctgtgatggccaggccaggggacccctgccagttctctgtggaagcaaggctg
gggtcttgggttcctgtatggtggaagctgggtgagccaaggacagggctggctcctctgcccccgctg
acgcttcccttgccgttggctttggatgtctttgctgcagtcttctctctggctcaggtgtgggtgggaggggc
ccacaggaagctcagccttctcctcccaaggtttgagtccctccaaagggcagtgggtggaggaccgg
gagctttgggtgaccagccactcaaaggaactttctggtcccttcagtatcttcaaggtttggaaactgca
aatgtccccttgatggggaatcc**gtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg**
ttttctcctagacccgtgacctgagatgtgtgatttttagtcattaaatggaagtgtctgccagctgggccca
gcacctaaaaaaaaaaaaaaaaaaa

Figure 1 – The 3′-UTR of the *mPGES-1* gene contains a $(GT)_{24}$ dinucleotide repeat. The sequence shown is the 3′-UTR region of the *mPGES-1* messenger RNA (accession: NM_004878.4). The $(GT)_{24}$ dinucleotide repeat, marked in bold, is located between positions 1665–1712. The forward and reverse primer sequences used to amplify the dinucleotide repeats region from the tumors, the corresponding normal mucosa samples, and the CRC cell line DNA samples are underlined.

3. Results

3.1. A GT dinucleotide repeat is located in the 3′-untranslated region (3′-UTR) of the human PTGES (mPGES-1) gene

While examining the 3′-UTR of the human *mPGES-1* gene for potential microRNA binding sites, we identified a $(GT)_{24}$ dinucleotide repeat region at position 1665-1712 of the mRNA (NM_004878.4) (Figure 1). This dinucleotide repeat region is not present in the mouse *Ptges* gene.

3.2. The $(GT)_{24}$ dinucleotide repeat region of the mPGES-1 gene exhibits instability in MSI-H colorectal tumors

Genetic inactivation or epigenetic inactivation (e.g., promoter hypermethylation of the MLH1 gene) of DNA mismatch repair (MMR) genes in human colorectal tumors leads to instability of microsatellite regions (Marra and Boland, 1995; Herman et al., 1998). Consequently, we examined the $(GT)_{24}$ dinucleotide region of the mPGES-1 gene for microsatellite instability in de-identified DNA samples from 13 MSS and 14 MSI-H human colorectal tumors, along with their corresponding normal mucosa. None of the 13 MSS tumors exhibited any change in the $(GT)_{24}$ repeat length, when compared to their matched normal mucosa (representative examples, Cases 1 and 2, Figure 2A–D). In contrast, changes in the $(GT)_{24}$ repeat length in the mPGES-1 genes of all 14 MSI-H tumors were observed relative to their matched normal mucosa (representative examples, Cases 3 and 4, Figure 2E–H), demonstrating instability of this $(GT)_{24}$ repeat in human MSI-H colorectal tumors. Elongation of the $(GT)_{24}$ repeat is present in ten of the fourteen MSI-H tumors (71%); the other four tumors (29%) have both shortened and elongated repeats (Table 1).

3.3. mPGES-1 mRNA levels are relatively higher in MSI-H CRC lines than in MSS CRC lines

Microsatellite instability in their 3′-UTRs are associated with altered expression of several genes in MSI-H colorectal tumors. (Paun et al., 2009; Yuan et al., 2009; Di Pietro et al., 2005) Ideally, we would want to examine the mPGES-1 mRNA levels in tumor samples, and/or monitor mPGES-1 protein levels by Western blotting or immunohistochemistry in the tumor samples from which our DNA samples were derived. However, because we initiated these studies with de-identified DNA samples from our clinical laboratory, we could not perform such analyses on the corresponding tumor tissues. To address the possibility that the *mPGES-1* $(GT)_{24}$ repeat might modulate mPGES-1 gene expression, we turned to CRC cell culture lines that had previously been characterized as MSS or MSI-H.

To determine whether $(GT)_{24}$ instability of the *mPGES-1* gene in MSI-H colorectal tumors is associated with altered *mPGES-1* gene expression we measured, by quantitative real time RT-PCR, *mPGES-1* mRNA levels in four reported MSI-H CRC lines (HCT116, LoVo, HCA7, and LS174T) and two reported MSS CRC lines (Caco2 and SW620). The characteristics of the cell lines are summarized in Table 2. Three of the four reported MSI-H CRC cell lines exhibited elevated *mPGES-1* mRNA levels when compared to the reported MSS CRC cell lines (Figure 3).

We next investigated whether *mPGES-1* mRNA levels in the CRC lines correlate with variations in their *mPGES-1* $(GT)_{24}$ repeat lengths. PCR amplification of the *mPGES-1*$(GT)_{24}$ repeat region from DNA isolated from the CRC lines, followed by fragment length analysis, showed differences in the $(GT)_{24}$ repeat length among these cell lines (Figure 4). The three MSI-H cell lines with the highest *mPGES-1* mRNA levels, HCT116, HCA7, and LoVo, showed both shortening and expansion of the $(GT)_{24}$ repeat, when compared to the other cell lines (Figure 4). Although LS174T cells are reported to be derived from an MSI-H tumor (Table 2), both *mPGES-1* mRNA levels and the *mPGES-1*$(GT)_{24}$ repeat characteristics of LS174T cells resemble those of the two cell lines reported to be derived from MSS tumors (Table 2). Taken together, these data suggest an association between the occurrence of $(GT)_{24}$ repeat instability and elevated *mPGES-1* mRNA levels in colorectal tumor cells.

4. Discussion

mPGES-1, a gene that encodes the terminal enzyme in the production of PGE_2, plays a role in CRC. (Wang and DuBois, 2010) The initial aim of this study was to determine whether mutations occur, in MSI-H CRCs, in the $(GT)_{24}$ dinucleotide repeat we identified in the 3′-UTR of the *mPGES-1* gene.

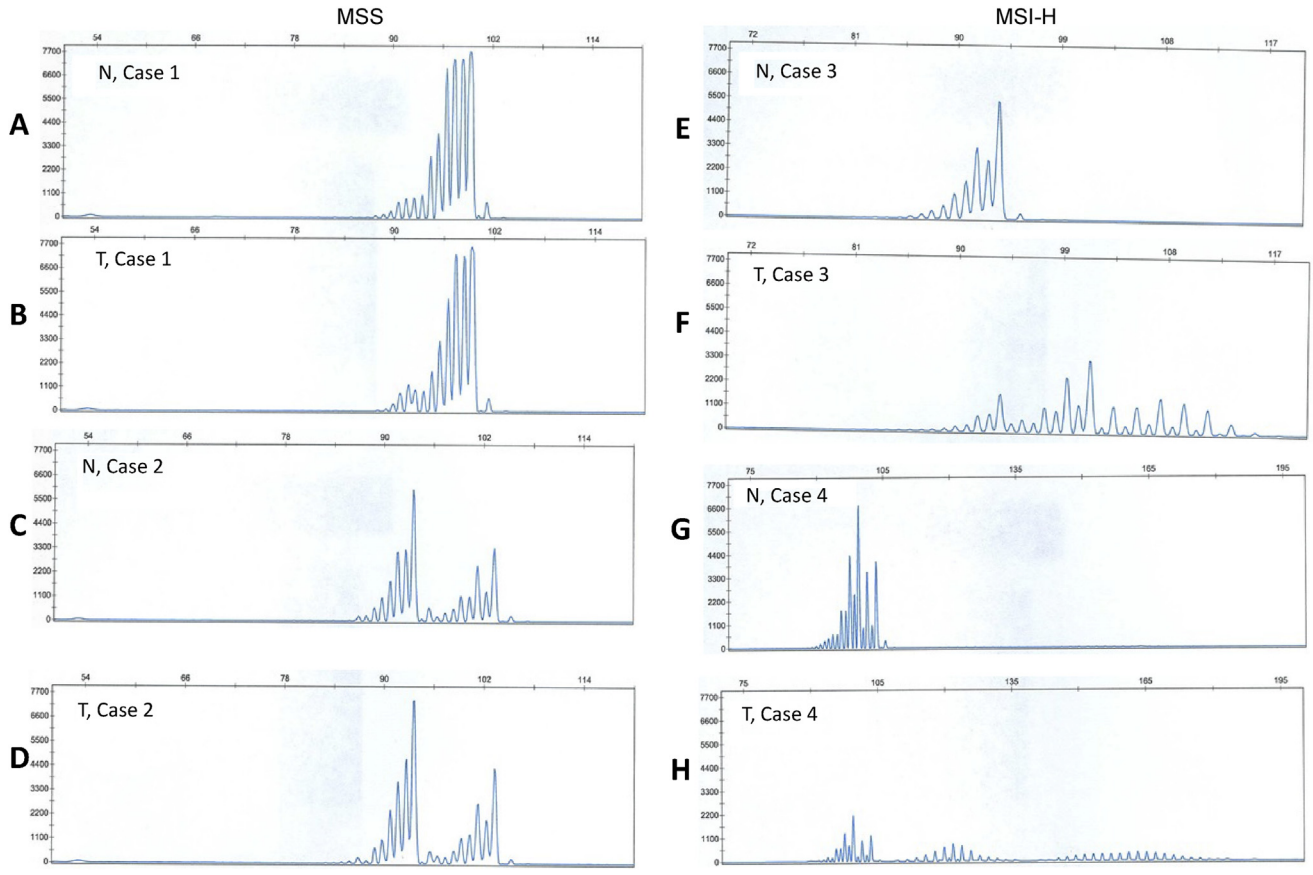

Figure 2 – Microsatellite instability analysis of the *mPGES-1*$(GT)_{24}$ repeat region in a series of human colorectal tumors. DNA samples from 13 MSS and 14 MSI-H CRC tumors and their matched normal mucosa were analyzed for $(GT)_{24}$ repeat stability. Electropherograms from two MSS tumors (T) and their control, normal mucosal samples (N) (A to D) and two cases of MSI-H (T) tumors and their control, normal mucosal samples (N) (E–H) are shown. A&B-MSS case 1; C&D-MSS case 2; E&F-MSI-H case 1; G&H-MSI-H case 2.

We observe mutations in the 3′-UTR *mPGES1*$(GT)_{24}$ repeat in the de-identified DNA samples from all 14 MSI-H colorectal tumors. In contrast, no alterations occur in the 3′-UTR *mPGES1*$(GT)_{24}$ repeat in the in the de-identified DNA samples from 13 MSS tumors analyzed. Among the fourteen MSI-H CRC tumors analyzed, most (10/14) cases showed only insertion mutations within the *mPGES-1*$(GT)_{24}$ repeat, resulting in elongation by as many as 37 repeat units. The other four MSI-H CRC tumors have both elongation and shortening mutations (Table 1). These results indicate *mPGES-1* is a target gene of microsatellite instability in human colorectal tumors. Notably, our results show 100% concordance between the *mPGES-1*$(GT)_{24}$ repeat instability and the data obtained with the NCI reference panel used clinically to distinguish MSI-H tumors. As stated by the NCI consensus panel, "Ideally, a given microsatellite sequence should demonstrate MSI frequently (e.g. >80% of the time) for a tumor defined as MSI-H but infrequently (e.g. <20% of the time) for a tumor in the MSI-L group". (Boland et al., 1998) The *mPGES-1*$(GT)_{24}$ repeat clearly exceeds these criteria and, therefore, appears to be a highly sensitive and specific marker in distinguishing MSI-H and MSS tumors in human CRC.

Table 1 – Allelic shift of $(GT)_{24}$ repeat observed in the MSI-H samples compared to the normal mucosa.

MSI-H sample	Allelic shift of (GT)24 repeat
1	Shortening and elongation
2	Elongation
3	Elongation
4	Elongation
5	Shortening and elongation
6	Shortening and elongation
7	Elongation
8	Elongation
9	Elongation
10	Elongation
11	Elongation
12	Elongation
13	Shortening and elongation
14	Elongation

Microsatellite sequence mutations located in regulatory regions have been reported previously in mismatch repair-deficient colorectal tumors. (Di Pietro et al., 2005) In MSI-H colorectal cancer genomes, elongation of either mono- or

Table 2 – Colorectal cancer cell lines and their MSS/MSI status.

Cell line	MSS/MSI status	Other information	References
HCA-7	MSI	MLH1 absent, MSH2 and MSH6 normal by IHC	Jung et al. (2009), [a] Williams et al. (2010)
HCT116	MSI	MLH1 absent, MSH2 and MSH6 normal by IHC; MLH1 absent, MSH2 and MSH6 normal, PMS2 very weak and unmethylated *MLH1* promoter	Di Pietro et al. (2005), Jung et al. (2009), Williams et al. (2010)
LoVo	MSI	MLH1 normal, MSH2 and MSH6 absent by IHC	Jung et al. (2009), Williams et al. (2010)
LS174T	MSI	MLH1 absent, MSH2 and MSH6 normal by IHC [16]; MLH1 very weak, MSH2 low, MSH6 low, PMS2 very weak, unmethylated *MLH1* promoter [14]	[b] Di Pietro et al. (2005), Jung et al. (2009), Williams et al. (2010)
Caco2	MSS	Normal MLH1, MSH2, MSH6, and PMS2 proteins; unmethylated *MLH1* promoter	Jung et al. (2009), Williams et al. (2010)
SW620	MSS	Normal MLH1, MSH2, MSH6, and PMS2 proteins; unmethylated *MLH1* promoter	Jung et al. (2009)

a Expression of MLH1, MSH2, and MSH6 proteins was determined by immunohistochemistry.
b Expression of MLH1, MSH2, MSH6, and PMS2 protein levels was determined by western blot analysis.

dinucleotide repeats has been observed more frequently in the 3′-UTR than in the coding regions. (Kim et al., 2013) Mutation in an A_{13} repeat in the 3′UTR of the *EGFR* gene, often present in MSI-H CRCs, results in increased expression from the *EGFR* gene. (Yuan et al., 2009) Similarly, mutations in the T_{10} mononucleotide repeat in the 3′UTR of the *RB1CC* gene correlate with up-regulation of *RB1CC* mRNA levels in MSI-H CRCs. (Paun et al., 2009) To rigorously address the relationship between the mPGES-1 $(GT)_{24}$ repeat and gene expression, it would be necessary to measure mPGES-1 mRNA levels and/or mPGES-1 protein levels in samples from the normal tissue and tumor tissue of the patients from which our DNA samples were derived. However, because our DNA samples were de-identified, this study could not be carried out.

To initially approach the functional impact of the $(GT)_{24}$ repeat instability on *mPGES-1* gene expression in MSI-H CRCs, we measured *mPGES-1* mRNA levels in human CRC lines of reported MSS and MSI-H status. Quantitative RT-PCR studies showed differential *mPGES-1* gene expression when comparing MSS and MSI-H CRC lines; higher *mPGES-1* mRNA levels were observed in three of the four CRC lines reported to have an MSI-H genotype (HCT116, LoVo, and HCA-7), compared to those CRC lines reported to have an MSS genotype (Figure 3). The HCT116, LoVo, and HCA-7 MSI-H CRC lines showed greater heterogeneity in their *mPGES-1*$(GT)_{24}$ repeat lengths, when compared to the mPGES-1$(GT)_{24}$ repeat lengths of the Caco2 and SW620 MSS CRC lines (Figure 4). These results can be explained by the lack of expression of MLH1, MSH2 or MSH6 proteins in HCT116, LoVo and HCA-7 cell lines (Table 2). In contrast, the *mPGES-1* mRNA level and the *mPGES-1*$(GT)_{24}$ repeat length observed in the LS174T cell line, also reported to be MSI-H (Table 2), were comparable to those observed in the two MSS cell lines Caco2 and SW620. This difference could be due to the presence of low levels of MLH1, MSH2, MSH6, and PMS2 proteins in LS174T cells (Di Pietro et al., 2005).

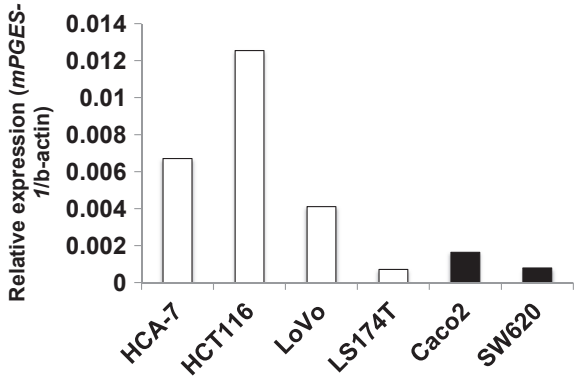

Figure 3 – *mPGES-1* mRNA expression in human colorectal cancer cell lines. Total mRNA was isolated from MSI-H (HCT116, LoVo, HCA7 and LS174T; open bars) and MSS (Caco2 and SW620, filled bars) CRC cell lines. cDNA synthesis was performed, and *mPGES-1* mRNA levels were determined by quantitative real time RT-PCR. Data are normalized to β-actin levels.

It is important to note that, despite the lack of conformity with the previous designation of LS174T as an MSI-H tumor cell line, we observe a complete correlation between the relative levels of *mPGES-1* mRNA (Figure 3) and the characteristics of the *mPGES-1*$(GT)_{24}$ repeat (Figure 4) in all six CRC cell lines. This observation emphasizes the value of having normal and tumor tissue for characterization of MSS and MSI-H status.

In conclusion, we demonstrate here that the *mPGES-1* gene is a previously unrecognized target of MSI in human CRC. Our data with de-identified DNA samples suggest the clinical utility of the mPGES-1$(GT)_{24}$ and our data with CRC cell culture lines suggest that mutation of the *mPGES-1*$(GT)_{24}$ sequence may modulate gene expression in colorectal cancers. However, confirmation and validation of these observations will require additional analyses of DNA samples of tumor tissue and corresponding mucosal samples of MSS and MSI-H patients, as well as *mPGES-1* mRNA and/or protein levels in these tissue samples. Additional studies are needed to evaluate the clinical validity and utility of the *mPGES-1*$(GT)_{24}$ repeat and its influence on *mPGES-1* gene expression.

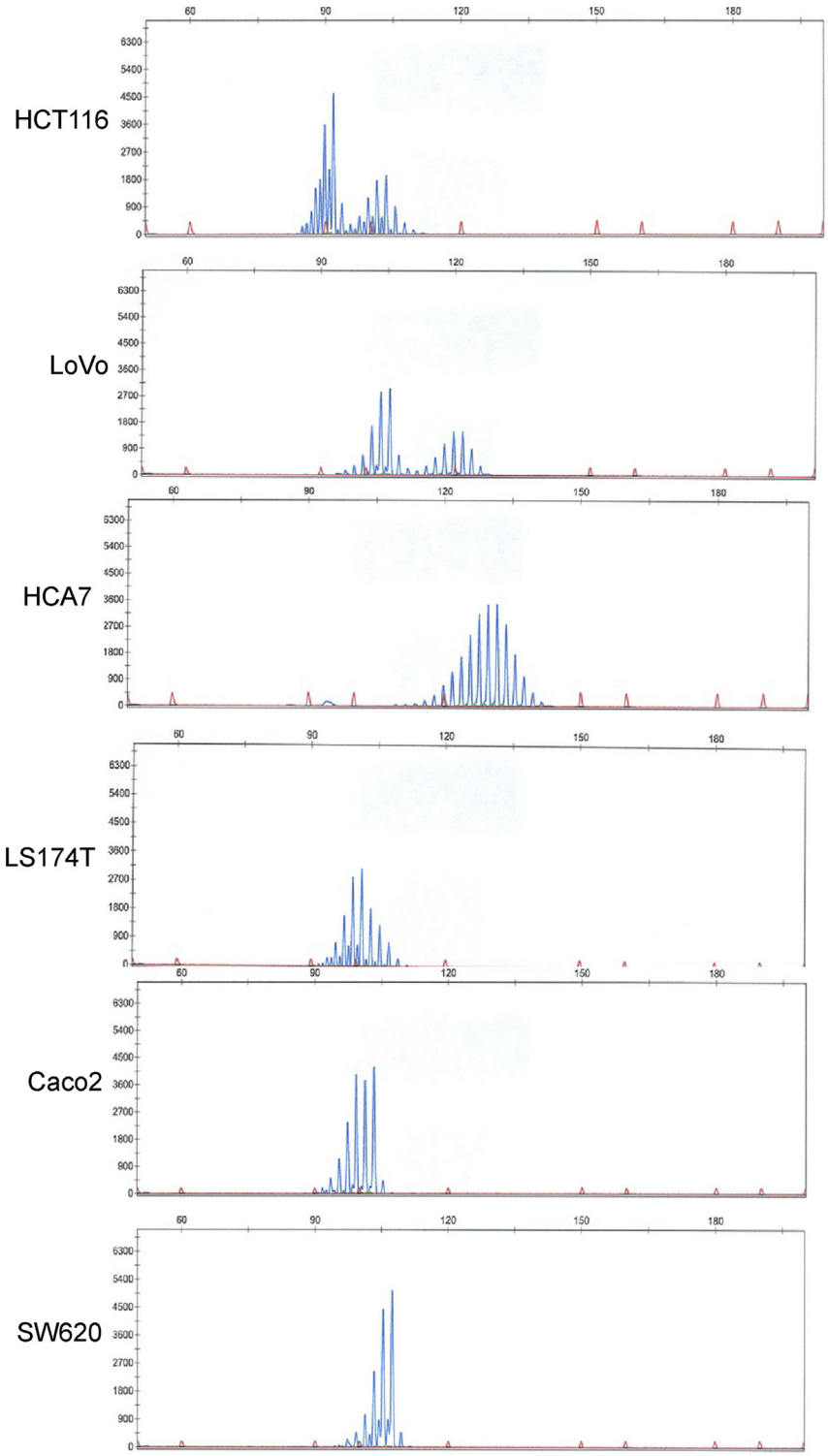

Figure 4 – Analyses of the *mPGES-1*$(GT)_{24}$ dinucleotide repeat region in human colorectal cancer cell lines. The $(GT)_{24}$ repeat regions amplified by PCR from DNA isolated from the human HCT116, LoVo HCA7, LS174T, Caco2 and SW620 CRC cell lines was subjected to capillary electrophoresis. Electropherograms for all CRC cell lines are shown.

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