

Research Article

Telomere Elongation and Mismatch Repair Deficiency Distinguish MSI Colorectal Cancer Cell Lines

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Abstract

Microsatellite instability (MSI) is a hallmark of mismatch repair (MMR) deficiency and characterizes a distinct subset of colorectal cancers (CRC). In parallel, telomere length dynamics have emerged as important contributors to genomic stability and tumorigenesis. However, the relationship between MSI status, MMR protein expression, and telomere maintenance remains poorly defined. This study aimed to investigate the association between MSI status and telomere length in CRC cell lines and to evaluate the expression of key MMR proteins (MLH1, MSH2, MSH6, PMS2) to elucidate molecular differences between MSI and microsatellite stable (MSS) phenotypes. A panel of CRC cell lines with known MSI and MSS statuses was used. Telomere length was quantified using real-time quantitative PCR (qPCR) based on the T/S ratio method. MSI status was confirmed via PCR using mononucleotide repeat markers. Western blotting was performed to assess protein expressions of MLH1, MSH2, MSH6, and PMS2. β -actin served as a loading control. qPCR analysis revealed that MSI cell lines exhibited significantly longer telomeres compared to MSS lines ($P < 0.05$). Western blot results showed complete loss of MSH6 protein expression and reduction in expression levels of PMS2 and MSH2 in MSI cell lines, confirming MMR deficiency. In contrast, MSS cell lines maintained normal expression of all tested MMR proteins. These findings suggest a link between defective MMR function and altered telomere dynamics in MSI-CRC. MSI CRC cell lines exhibit telomere elongation and loss of key MMR proteins, highlighting distinct molecular features compared to MSS counterparts. These insights may inform future strategies for personalized CRC diagnostics and therapeutics, particularly in the context of telomere-targeted or immunomodulatory treatments.

Keywords

Colorectal Cancer, Telomere Length, Microsatellite Instability, Mismatch Repair Pathway, MMqPCR, Western Blotting, HRM Analysis

1. Introduction

Colorectal cancer (CRC) ranks as the third most common cancer globally and is the second leading cause of cancer-related mortality, with an estimated 1.9 million new cases and 935,000 deaths in 2020 [1]. Although CRC primarily

affects individuals over the age of 50, an alarming rise in early-onset CRC has been noted in younger populations. The disease generally evolves from benign adenomatous or serrated polyps, progressing via a multi-step carcinogenic se-

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quence driven by cumulative genetic and epigenetic alterations. The adenoma-carcinoma sequence, first conceptualized by Fearon and Vogelstein, continues to serve as a foundational framework for elucidating CRC pathogenesis [2].

A hallmark of CRC is genomic instability, which manifests through three major and often overlapping mechanisms: chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) [3]. These molecular pathways not only fuel tumor heterogeneity and progression but also significantly influence therapeutic resistance [4].

1.1. Microsatellite Instability in CRC

Microsatellite instability (MSI) is defined by the accumulation of length variations in short tandem repeats due to defective DNA mismatch repair (MMR) [4]. First reported in CRC in the early 1990s, MSI is present in approximately 15-20% of sporadic CRCs and more than 95% of Lynch syndrome-associated tumors [4, 5]. MSI is classified into three categories—MSI-high (MSI-H), MSI-low (MSI-L), and microsatellite stable (MSS)—based on the frequency and pattern of instability [5]. MSI-H tumors are associated with high tumor mutational burden (TMB), enhanced neoantigen presentation, better prognosis, and improved response to immune checkpoint inhibitors [6].

1.2. Mismatch Repair (MMR) Pathway and CRC

The MMR pathway is vital for maintaining genomic fidelity by correcting base-base mismatches and insertion-deletion loops during DNA replication. Core MMR proteins include MLH1, PMS2, MSH2, and MSH6, which operate as heterodimers (e.g., MutS α , MutL α) to recognize and repair replication errors [7]. In sporadic CRC, MSI is frequently attributed to the epigenetic silencing of the MLH1 gene through promoter hypermethylation [8]. Dysfunctional MMR leads to an accumulation of somatic mutations, thereby disrupting oncogenes and tumor suppressor pathways [9].

1.3. Telomere Dysfunction and Its Link to MMR

Telomeres, composed of tandem TTAGGG repeats, protect chromosomal termini from degradation and fusion. Progressive telomere shortening, a natural consequence of cellular replication, can induce senescence or apoptosis [10]. In CRC, telomere attrition contributes to chromosomal instability and tumor evolution. MSI-H tumors have been shown to possess shorter telomeres than MSS counterparts, suggesting a correlation between MMR deficiency and telomere dynamics [11]. Moreover, loss of MMR proteins such as MLH1 and MSH2 promotes homologous recombination at telomeres, potentially enabling the alternative lengthening of telomeres (ALT) pathway [12].

1.4. Telomerase and ALT in CRC

Telomerase, a ribonucleoprotein complex composed of the catalytic subunit hTERT and the RNA component hTERC, is reactivated in nearly 90% of human cancers, including CRC [13]. In contrast, a subset of tumors maintains telomeres via the telomerase-independent ALT mechanism, which relies on homologous recombination [14]. Intriguingly, MSI-H CRCs frequently exhibit diminished hTERT expression, indicating potential reliance on ALT for telomere maintenance [15]. Therefore, exploring the interactions between MMR deficiency and telomere regulation may offer novel insights into CRC biology and therapeutic targets.

2. Materials and Methods

2.1. Cell Culture

Human CRC cell lines CACO2 (MSS, RRID: CVCL_0025) and HCT15 (MSI, RRID: CVCL_0292) were procured and cultured under standard conditions at 37 °C in a humidified 5% CO₂ atmosphere. CACO2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), while HCT15 cells were grown in RPMI 1640 medium with 10% FBS. All protocols adhered to ATCC guidelines [16].

2.2. DNA and Protein Extraction

Cells were harvested at ~90% confluence using trypsin-EDTA. Genomic DNA was extracted by phenol-chloroform method [17], verified for integrity via 1% agarose gel electrophoresis, and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Protein extraction was performed using RIPA buffer, followed by quantification and analysis according to standard protocols [18].

2.3. Telomere Length Measurement

Telomere length was assessed using monochrome multiplex quantitative real-time PCR (MMqPCR) as described by Cawthon [20, 21] and refined by Hosen et al [19]. Primer sequences were:

Telomere primers:

TelG: ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT

TelC: TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTATCCCTAACA

Single-copy gene (albumin) primers:

AlbUgr2: CGGCGGCGGGCGGCGGCGGGCTGGGCGCGGCGGCGGGCGGCGGCGGGCTGGGCGGAAATGCTGCACACAGAATCCTTG

AlbDgr2:

GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAA-GCATGGTCGCCTGTT

Reactions were performed using the QuantStudio 6 Pro Real-Time PCR system. T/S ratios (telomere repeat copy number to single-copy gene number) were calculated from Ct values to estimate relative telomere length [20].

2.4. Microsatellite Instability Detection

MSI status was determined using high-resolution melting (HRM) analysis adapted from Raji et al [21]. The pentaplex panel included five mononucleotide markers [22]:

NR-27: F: AACCATGCTTGCAAACCACT | R: CGA-TAATACTAGCAATGACC

NR-21: F: GAGTCGCTGGCACAGTTCTA | R: CTGGTCACTCGCGTTTACAA

NR-24: F: GCTGAATTTTACCTCCTGAC | R: ATT-GTGCCATTGCATTCCAA

BAT-25: F: TACCAGGTGGCAAAGGGCA | R: TCTGCATTTTAACTATGGCTC

BAT-26: F: CTGCGGTAATCAAGTTTTTAG | R: AACCATTCAACATTTTAAACCC

qPCR amplification was followed by HRM profiling to detect heteroduplexes indicative of MSI [23].

2.5. Western Blotting

Protein concentrations were determined using the BCA assay [24]. Lysates were electrophoresed on SDS-PAGE and transferred to membranes via semi-dry transfer. Membranes were blocked with 5% BSA or milk, then probed overnight at

4°C with primary antibodies against hMLH1, hMSH2, hMSH6, hPMS2, and β -actin [25]. HRP-conjugated secondary antibodies were used, and bands were visualized using DAB or luminol substrate. Images were captured using ImageQuant software.

3. Results

3.1. DNA Quality Assessment and Quantification

For the MSI HCT15 cell line, the DNA showed high purity with A260/A280 ratios ranging between 1.80 and 1.90, and DNA yields were consistently above 100 ng/ μ L. Similarly, MSS CACO2 DNA samples demonstrated excellent integrity, with no smearing observed on gels and comparable purity metrics. These results confirmed that the genomic DNA was of suitable quality for downstream applications such as qPCR and HRM analyses.

3.2. Telomere Length Estimation

MMqPCR analysis revealed a significantly reduced telomere length (T/S ratio) in MSI-H HCT15 cells compared to MSS CACO2 cells ($p < 0.05$). The mean T/S ratio in HCT15 was 0.71 ± 0.08 , whereas CACO2 cells exhibited a higher T/S ratio of 1.26 ± 0.11 (Figure 1). This indicates accelerated telomere shortening in MSI-H cells, aligning with the hypothesis that MMR deficiency may contribute to telomere erosion.

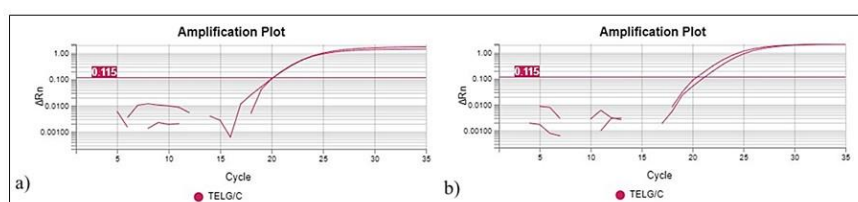


Figure 1a) Amplification plot: Telomere length measurement using TelG/C primers
a)HCT15 (MSI) CRC Cell line; b)CACO2 (MSS) CRC cell line. Telomere-specific primers amplify telomeric repeat regions to quantify relative telomere length. The difference in amplification curves indicates differential telomere content between MSI and MSS cell lines.

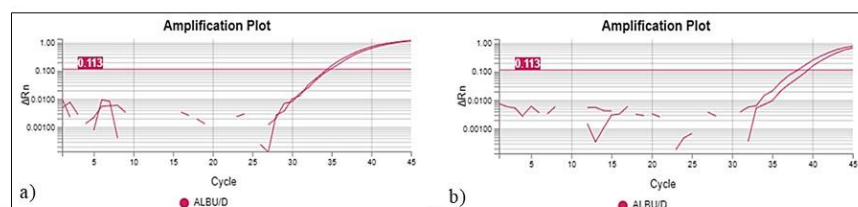


Figure 1b) Amplification plot: Telomere length measurement using AlbU/D primers
a)HCT15 (MSI) CRC cell line; b)CACO2 (MSS) CRC cell line. The AlbU/D primers target a single-copy gene (albumin) to serve as normalization control in relative telomere length calculations.

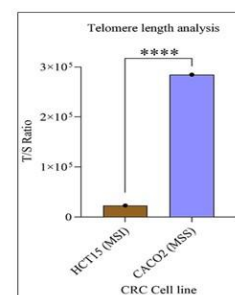


Figure 1c) Comparison of Telomere Length in MSI and MSS CRC Cell lines. Relative telomere length, expressed as the telomere/single-copy gene (T/S) ratio, was significantly lower in MSI cell line (HCT15) compared to MSS cell line (CACO2), indicating pronounced telomere attrition (**** $P < 0.0001$). Data are presented as mean $\pm$ SD from three independent experiments

Figure 1. Telomere length Analysis.

Table 1. Telomere Length Estimation in CRC Cell Lines via T/S Ratio Relative telomere length (T/S ratio) in HCT15 (MSI) and CACO2 (MSS) cell lines was determined using the ΔC_t method ($C_{tT} - C_{tS}$) and calculated as $2^{\Delta C_t}$. C_t values were obtained via qPCR using TelG/C (telomeric) and AlbU/D (single-copy) primers. SD reflects variability across replicates.

Cell Line	C_{tT} (TelG/C)	C_{tS} (AlbU/D)	T/S Ratio ($2^{\Delta C_t}$)	SD
HCT15 (MSI)	20.13	34.61	22,862.18	1.28
CACO2 (MSS)	20.60	38.72	284,641.89	1.63

3.3. Microsatellite Instability Analysis

HRM profiles generated from the five-marker pentaplex assay (BAT-25, BAT-26, NR-21, NR-24, and NR-27) showed significant heteroduplex formation and distinct melting curve

shifts in HCT15, confirming its MSI-H status. In contrast, CACO2 cells showed stable melting profiles across all five markers, consistent with an MSS phenotype (Figure 2). These findings validate the suitability of HRM as a rapid, dye-based, high-throughput method for MSI detection in CRC cell lines.

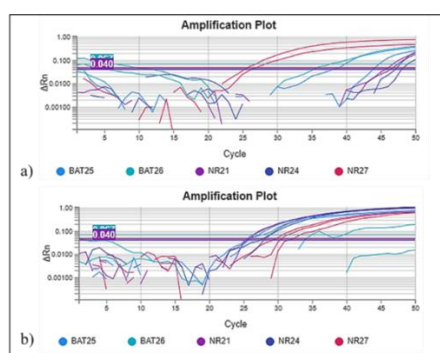


Figure 2a). Amplification of Microsatellite Markers in CRC cell lines. Representative gel images showing microsatellite region amplification in a) HCT15 (MSI) and CACO2 (MSS) CRC cell lines using pentaplex panel of markers (NR21, NR24, NR27, BAT25, BAT26). Multiple shifts and instabilities were observed in HCT15, confirming its MSI-H phenotype.

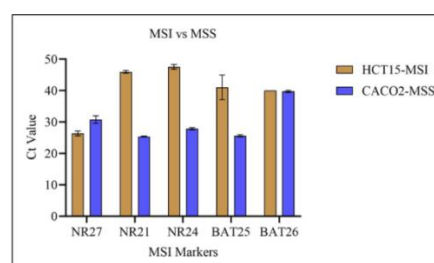


Figure 2b). High Resolution Melting (HRM) Analysis for MSI detection. Detection of Microsatellite Instability in CRC cell lines using HRM with a pentaplex panel. Distinct melting profiles were observed for HCT15 (MSI) compared to CACO2 (MSS), with marker-specific shifts in melting temperature confirming MSI status in HCT15 cell line.

Figure 2. Microsatellite Instability Analysis.

Table 2. MSI Marker Amplification in CRC Cell Lines by HRM-qPCR Mean C_t values and SDs for five MSI markers analyzed via HRM-qPCR in HCT15 (MSI) and CACO2 (MSS) cells. C_t shifts indicate differential amplification efficiency related to MSI status.

MSI Marker	HCT15 (MSI) - Mean C_t	SD	CACO2 (MSS) - Mean C_t	SD
NR27	26.37	0.77	30.80	1.18
NR21	45.97	0.46	25.42	0.08
NR24	47.58	0.75	27.87	0.35
BAT25	41.05	3.95	25.62	0.37
BAT26	40.00	0.00	39.76	0.35

3.4. Western Blotting of MMR Proteins

Western blot analysis revealed a complete loss of MSH6

protein expression in the MSI-H HCT15 cell line, whereas MSH6 was robustly expressed in the MSS CACO2 cells. The expression levels of MSH2 and PMS2 were also significantly reduced in HCT15 cells, indicating downstream destabiliza-

tion of the MMR heterodimers due to the absence of MSH6. Interestingly, MLH1 expression showed a 1.7-fold increase in the MSI cell line, suggesting a potential compensatory re-

sponse. β -actin expression remained consistent across both cell lines, confirming equal protein loading (Figure 3).

Table 3. Protein Quantification in CRC Cell Lysates via BCA Assay Total protein concentrations ($\mu\text{g/mL}$) in CACO2 and HCT15 lysates were determined using the BCA assay. Absorbance at 562 nm was blank-corrected and values calculated using a standard curve. Measurements were performed in duplicate.

Sample	Absorbance (562 nm)	Blank	Protein Concentration ($\mu\text{g/mL}$)
CACO2_P1	1.2322	0.9717	3190
CACO2_P2	1.3291	1.0686	3513
HCT15_P1	1.5952	1.3347	4400
HCT15_P2	1.6422	1.3817	4556.5

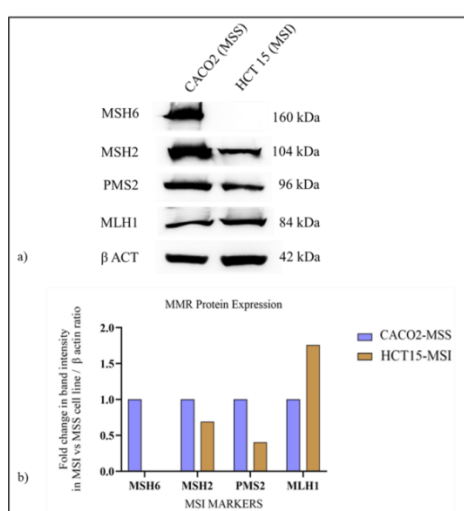


Figure 3. Western Blot Analysis of MMR Protein Expression.

Expression levels of key MMR Proteins (MSH6, MSH2, PMS2, MLH1) in MSI (HCT15) and MSS (CACO2) CRC cell lines. MSI cells showed absent MSH6, reduced MSH2 and PMS2, and upregulated MLH1 expressions. β -actin was used as a loading control

Figure 3. MMR Protein expression analysis.

4. Discussion

Colorectal cancer (CRC) pathogenesis involves diverse molecular subtypes, with microsatellite instability (MSI) and telomere dysfunction being pivotal contributors. This study demonstrates a significant correlation between MSI status and telomere shortening, coupled with downregulation of DNA mismatch repair (MMR) proteins, suggesting a mechanistic interplay between genomic instability and telomere biology in CRC. Our findings contribute to a growing body of evidence implicating the involvement of MSI and telomere dysfunction in CRC development.

MMR Deficiency and MSI in CRC: Our HRM-based MSI assay effectively differentiated between MSI-H (HCT15) and MSS (CACO2) phenotypes. This was in agreement with previous reports that highlight the critical role of MMR proteins in maintaining genomic stability [26, 27]. The analysis re-

vealed that MSH6 was not expressed in the MSI cell line, and there was downregulation of MSH2 and PMS2. Conversely, MLH1 expression was upregulated. These results suggest that the MSI cell line's heightened mutational burden and disrupted telomere maintenance may be due to the compromised functionality of specific MMR proteins [28, 29]. In particular, this study highlights that the absence of MSH6 protein expression is a significant contributor to microsatellite instability (MSI) in the CRC cell line. This deficiency not only induces MSI due to the inability to repair MMR mismatches but also appears to play a crucial role in the accelerated telomere shortening observed in MSI CRC cell lines. The simultaneous loss or reduced expression of MSH6, PMS2, and MSH2 likely compromises DNA repair efficiency, thereby exacerbating genomic instability. The elevated MLH1 expression in the MSI cell line may indicate a compensatory response to the loss of other MMR proteins, although its precise role in this context requires further investigation. The general classification and characterization of MSI across various types of

cancer have been systematically outlined in previous studies, and de la Chapelle (2003) provided the foundational understanding of MSI's role in CRC [30].

Telomere Shortening in MSI-H CRC: Our data on telomere length reveals a significant reduction in telomere size in HCT15 cells, in agreement with previous studies suggesting that MSI-H tumors tend to harbor shorter telomeres compared to their MSS counterparts [31, 32]. This reduction in telomere length could be attributed to the loss of MMR function, which normally prevents aberrant homologous recombination at telomeres. The absence of functional MMR proteins likely leads to the destabilization of telomeres, promoting telomere attrition in MSI-H tumors. Moreover, the observed reduction in telomere length aligns with the hypothesis that MSI-H tumors may rely on alternative telomere lengthening (ALT) mechanisms in the absence of telomerase reactivation, a phenomenon well-documented in MMR-deficient cancers [33]. These findings align with the systematic analysis of telomere lengths and somatic alterations in CRC, which reveal a close association between telomere attrition and tumor progression [30].

Therapeutic and Diagnostic Implications: The findings of this study support the growing paradigm that MSI status and telomere dynamics can be leveraged for CRC prognosis and therapy. MSI-H tumors, due to their high neo-antigen burden, are known to respond favorably to immune checkpoint blockade therapies [34]. Understanding the interplay between telomere dysfunction and MSI may open new avenues for targeted therapeutic strategies. For example, telomere-based diagnostics could be utilized to stratify patients for telomerase-targeted or ALT-inhibitory therapies, both of which may hold promises for improving clinical outcomes in MSI-H CRC patients.

Limitations and Future Directions: While this study provides robust in vitro data, it is limited to only two cell lines, which may not fully capture the complexity of CRC heterogeneity. Future work should aim to include primary tumor samples from MSI-H and MSS CRC patients to validate these observations and assess their clinical relevance. Additionally, telomere length assays, telomerase activity assays, and ALT pathway analyses could provide further insights into the telomere maintenance strategies employed by different CRC subtypes. Expanding the study to encompass a broader range of CRC models will help to define the full extent of telomere dysfunction and its therapeutic implications in MSI-associated cancers.

Abbreviations

CRC	Colorectal Cancer
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability - High
MMR	Mismatch Repair
MSS	Microsatellite Stable
CIN	Chromosomal Instability

CIMP	CpG island Methylator Phenotype
ALT	Alternative Lengthening of Telomeres
MMqPCR	Monochrome Multiplex Quantitative Real-Time PCR
T/S Ratio	Telomere Repeat Copy Number to Single-Copy Gene Number
HRM	High-Resolution Melting

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Author Contributions

Rajasekhar Moka: Conceptualization, Writing-Review and Editing, Supervision.

Shreya Upadhyia: Investigation, Methodology, Visualization, Validation, Formal Analysis, Writing-Original Draft.

Conflicts of Interest

The authors declare no conflicts of interest.

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