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# Genomic Instability Causes HGF Gene Activation in Colon Cancer Cells, Promoting Their Resistance to Necroptosis

Danushka Seneviratne,* Jihong Ma,* Xinping Tan, Yong-Kook Kwon, Eman Muhammad, Mona Melhem, Marie C. DeFrances, and Reza Zarnegar*

*Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania*

**BACKGROUND & AIMS:** Genomic instability promotes colon carcinogenesis by inducing genetic mutations, but not all genes affected by this process have been identified. We investigated whether genomic instability in human colorectal cancer (CRC) cells produces mutations in the hepatocyte growth factor (*HGF*) gene. **METHODS:** We genotyped human colon tumor tissues and adjacent nontumor tissues collected from 78 patients University of Pittsburgh Health Sciences and Veterans Hospital, along with 40 human CRC and adjacent nontumor tissues in a commercial microarray. We used cellular, biochemical, and molecular biological techniques to investigate the factors that alter HGF signaling in colon cancer cells and its effects on cell proliferation and survival. **RESULTS:** All tested human CRC tissues and cell lines that had microsatellite instability contained truncations in the regulatory deoxyadenosine tract element (DATE) of the *HGF* gene promoter. The DATE was unstable in 14% (11 of 78) of CRC samples; DATE truncation was also polymorphic and detected in 18% (13 of 78) of CRC tissues without microsatellite instability. In CRC cell lines, truncation of DATE activated expression of HGF, resulting in its autocrine signaling via MET. This promoted cell proliferation and resistance to necroptosis. HGF signaling via MET reduced levels of the receptor-interacting serine-threonine kinase 1, a mediator of necroptosis, in CRC cells. High levels of HGF protein in tumor tissues correlated with lower levels of receptor-interacting serine-threonine kinase 1 and shorter survival times of patients. **CONCLUSIONS:** Thirty-one percent of CRC samples contain alterations in the DATE of the *HGF* promoter. Disruption of the DATE increased HGF signaling via MET and reduced levels of receptor-interacting serine-threonine kinase 1 and CRC cell necroptosis. DATE alteration might be used as a prognostic factor or to select patients for therapies that target HGF–MET signaling.

*Keywords:* Signal Transduction; Receptor Tyrosine Kinase; Oncogene; Gene Regulation.

Hepatocyte growth factor (HGF) induces complex intracellular signaling networks resulting in cell proliferation and cell survival, leading to regeneration and homeostasis of various epithelial tissues. HGF exerts its biologic effects via its tyrosine kinase cell surface receptor known as Met.[1–4] When aberrantly activated, however, the HGF–Met pathway could act as an oncogenic "driver" and promote tumorigenesis.[1,2] Stromal but not epithelial cells normally produce HGF, and Met is highly expressed in epithelial cells, creating a paracrine loop between stromal and epithelial cells.[1–3] *HGF* gene transcription is silenced in normal epithelial cells, and its expression in stromal cells is tightly regulated.[5–8] We recently showed that *HGF* gene transcription is activated in human breast carcinomas due to mutation in a novel regulatory *cis*-acting element located approximately 700 bp from *HGF*'s basal promoter. The mutation causes HGF expression and establishment of an HGF-Met autocrine circuit in breast carcinoma cells. This element, which we have named deoxyadenosine tract element (DATE), consists of a polyA tract of 30A, represses *HGF* gene transcription in epithelial cells by modulating chromatin structure and binding transcriptional repressors and activators to HGF promoter.[9] The molecular basis of DATE mutagenesis, as well as the molecular consequences of aberrant HGF expression remained unknown. More importantly, it was also unknown whether DATE mutation occurs in other forms of human carcinomas. Given the fact that colorectal carcinoma (CRC) is currently one of the most common forms of cancer in developed nations, with an estimated annual global mortality of 529,000,[10] we designed experiments to test our hypothesis that the *HGF* gene could be a target of mutagenesis in human CRC.

## Methods

Archival human colon tumor tissues and their corresponding normal adjacent tissues from 78 patients were obtained from the University of Pittsburgh Health Sciences Tissue Bank and Veteran's Affairs Hospital, according to an approved Institutional Review Board. An additional 40 human CRC cases and their corresponding adjacent normal tissues in the form of a tissue microarray (IMH-359) were purchased from IMGENEX (Littleton, CO). Information regarding the tissue microarray is provided at: http://www.imgenex.com. All CRC cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured according to the supplier's instructions.

*Authors share co-first authorship.

*Abbreviations used in this paper:* CRC, colorectal carcinoma; DATE, deoxyadenosine tract element; HGF, hepatocyte growth factor; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable.


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### Analysis of Deoxyadenosine Tract Element and Microsatellite Instability Status

Genomic DNA was prepared from human cell lines and tissues using TRIzol reagent (Invitrogen, Carslbad, CA) according to the manufacturer's instructions. DATE region was amplified by polymerase chain reaction according the to the protocol described.[9] The sequences of the fluorescent labeled 5′-FAM primers (made by Integrated DNA Technologies, Coralville, IA) used to amplify the DATE fragment are as follows: forward primer 5′–TATTTCGTGAGTTTGGCAGTTTG TG−3′ and reverse primer; 5′−AACAAAAGCACGCAGATTGTC AGATG−3,′ which will yield a 121-bp DNA product. For microsatellite instability (MSI) determination, we used an assay kit from Promega (Madison, WI), which contain labeled primers for 7 markers; 5 mononucleotide repeat markers: NR-21 (SLC7A8; 103 bp), BAT-25 (c-kit; 124 bp), BAT-26 (hMSH2; 115 bp), NR-24 (ZNF-2; 132 bp), and MONO-27 (152bp) and 2 Penta repeats; Penta C and Penta D. The system allows amplification and detection of all markers in a single reaction. All polymerase chain reaction products were separated by capillary electrophoresis using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Data analysis was performed using the GeneScan Analysis software. MSI status was determined by assessing each of the mononucleotide markers for instability. Samples were classified as being either MSI-high if at least 2 of the 5 markers showed instability, MSI-low if 1 marker showed instability, and microsatellite stable (MSS), in the absence of instability.

### Statistical Analyses

Two-tailed Student *t* test and Fisher's exact test were used to analyze data as indicated. To determine survival differences, Kaplan-Meier curves were generated for overall and stage-adjusted 5-year survival rates using GraphPad software. Patients who died of causes unrelated to cancer were excluded from the computation. Log-rank (Mantel-Cox) test was used to calculate data significance and to determine the hazard ratio. $P$ values $\leq .05$ were considered to be significant in all statistical analyses. Significant differences between the groups corresponding to $P < .05, .01, .001$, and not significant are depicted by *, **, ***, and NS, respectively.

Additional information on methods can be found in Supplementary Materials and Methods.

## Results

### Deoxyadenosine Tract Element Deletion Mutagenesis Occurs in Human Colon Carcinomas as a Result of Defective DNA Mismatch Repair System

We genotyped human sporadic CRC tissues for DATE status and discovered that DATE is unstable and undergoes deletion (truncation) mutagenesis in the tumors as compared with corresponding matched adjacent normal colon tissues (Figure 1*A*). DATE instability occurred at a frequency of 14% (11 of 78 cases). Notably, we found that DATE is polymorphic in nature as 17% (13 of 78) of cases harbored truncated DATE variant in the tumor samples as well as in their corresponding normal tissues (Figure 1*B*), suggesting that the truncated DATE variant is heritable. Overall, 24 of 78 (31%) of sporadic CRC cases had the truncated variant of DATE (Supplementary Table 1). We verified the polymorphic nature of DATE truncation using genomic DNA isolated from lymphocytes of healthy individuals and found that truncated DATE variant exists in the normal population (Supplementary Figure 1*A*) at a frequency of 7.7% (46 of 592). We compared the frequency of truncated DATE variant in CRC vs normal population and found that DATE truncation significantly associates with the incidence of CRC ($P < .0001$, 2- tailed Fisher's exact test). As DATE resembles a microsatellite, its mutation in the tumor could be due to MSI provoked by deficient mismatch repair (MMR). Accordingly, we analyzed the CRC cases that exhibited truncated DATE variant for MSI using the well-established NCI-recommended MSI marker panel, which consists of 5 mononucleotide repeat markers, NR-21, NR-24, BAT25, BAT26, MONO27 and 2 pentanucleotide repeats. The results revealed that nearly all of the cases with unstable DATE in the tumor exhibited the MSI-high phenotype, except for 2 cases, which were classified as MSS according to the MSI markers, but had unstable DATE (Figures 1*C*−*E*, and Supplementary Table 2) present only in the tumor and not in the adjacent normal tissue and are discussed in the Results section. In human CRC, MMR deficiency is most often due to the loss of "caretaker" gene function, *MLH1* or *MSH2*, therefore, the expression of these is routinely assessed clinically by immunohistochemistry for determination of MMR status. We performed immunohistochemistry and the data clearly showed that all DATE unstable tumors either lack MLH1 or MSH2 protein, and DATE stable tumors have robust expression of both (Figure 1*D*). The 2 DATE mutant tumor cases mentioned that were classified as MSS but showed DATE instability exhibited defective MLH1 protein expression (Figure 1*E*). Next, we studied 7 human colon carcinoma cell lines, which have known MMR profiles and MSI status for DATE alterations. Genotyping and sequencing results for DATE revealed that human CRC cell lines with MMR deficiency and MSI have truncated DATE: HCT116 (18A, 21A), HCT-15 (23A), RKO (18A), SW-48 (18A, 23A), and LoVo (17A). In contrast, wild-type DATE was observed in the 2 MSS and MMR-proficient cell lines, HT-29 (30A) and SW-620 (30A) (Figure 1*F* and Supplementary Figures 1*B* and *C*). We also assessed the MSI status of other human cancer cell lines with truncated DATE, namely C33A, a cervical carcinoma cell line, which has DATE with 12A, and Jurkat cells, a lymphoma cell line that has DATE with 16A. The data revealed that these cell lines with truncated DATE also have MSI (Supplementary Table 3).

### Deoxyadenosine Tract Element Truncation Causes Reactivation of the Hepatocyte Growth Factor Promoter Leading to Autocrine Production of Hepatocyte Growth Factor in Colorectal Cancer

Our next aim was to examine the functional consequences of DATE truncation on HGF promoter activity.