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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, PA 15261

Abstract

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 non-tumor tissues collected from 78 patients University of Pittsburgh Health Sciences and Veterans hospital, along with 40 human CRC and adjacent non-tumor 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 (MSI) contained truncations in the regulatory deoxyadenosine tract element (DATE) of the *HGF* gene promoter. The DATE was unstable in 14% (11/78) of CRC samples; DATE truncation was also polymorphic, and detected in 18% (13/78) of CRC tissues without MSI. 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 (RIPK1), a mediator of necroptosis, in CRC cells. High levels of HGF protein in tumor tissues correlated with lower levels of RIPK1 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

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^{*}To whom correspondence should be addressed: rezazar@pitt.edu, Tel.: 412-648-8657; Fax: 412-648-1916.

[#]The authors contributed equally to this work

Authors' contribution: R. Zarnegar developed the hypothesis, designed the experiments, analysed the data. D. Seneviratne, and J. Ma performed experiments, collected and analyzed the data, Xin-Ping Tan and Yong-Kook Kwon performed experiments. D. Seneviratne and R. Zarnegar wrote the manuscript. M. Melhem, and Eman Muhammad, certified Pathologists, collected human CRC tissues and M. C. DeFrances, also a certified Pathologist, participated in result interpretation.

Conflict of Interest: NONE

Note that authors names in bold designate shared co-first authorship.

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RIPK1 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

Introduction

HGF (Hepatocyte Growth Factor) induces complex intracellular signaling networks resulting in cell proliferation and cell survival, hence leading to regeneration and homeostasis of various epithelial tissues. HGF exerts its biological effects via its tyrosine kinase cell surface receptor known as Met¹⁻⁴. 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, whereas Met is highly expressed in epithelial cells, thus creating a paracrine loop between stromal and epithelial cells¹⁻³. *HGF* gene transcription is silenced in normal epithelial cells, and its expression in stromal cells is tightly regulated⁵⁻⁸. 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 DATE (DeoxyAdenosine Tract Element) 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 promoter⁹. 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 among developed nations, with an estimated annual global mortality of 529,000¹⁰, we designed experiments to test our hypothesis that the *HGF* gene could be a target of mutagenesis in human CRC.

Materials and 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 VA hospital, according to an approved IRB. 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. Information regarding the tissue microarray is provided in the following website: <http://www.imgenex.com>. All CRC cell lines were purchased from American Type Culture Collection (ATCC) and cultured according to the supplier's instructions.

Analysis of DATE and MSI status

Genomic DNA was prepared from human cell lines and tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. DATE region was amplified by PCR according to the protocol described⁹. The sequences of the fluorescent labeled 5'-

FAM primers (made by Integrated DNA Technologies) used to amplify the DATE fragment are as follows: forward primer 5'-TATTTCTGTGAGTTTGGCAGTTTGTG-3' and reverse primer; 5'-AACAAAAGCACGCAGATTGTCAGATG-3' which will yield a 121 bp DNA product. For MSI determination, we used an assay kit from Promega which contain labeled primers for seven markers; five 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 two Penta repeats; Penta C and Penta D. The system allows amplification and detection of all markers in a single reaction. All PCR products were separated by capillary electrophoresis using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). 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 (MSI-H) if at least two of the five markers showed instability, MSI-low (MSI-L) if one marker showed instability, and MSI-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 five-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 equal or less than 0.05 were considered to be significant in all statistical analyses. Significant differences between the groups corresponding to $P < 0.05$, 0.01, 0.001, and no difference are depicted by *, **, ***, and NS, respectively.

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

Results

DATE deletion mutagenesis occurs in human colon carcinomas as a result of defective DNA Mismatch Repair (MMR) 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 to corresponding matched adjacent normal colon tissues (Figure 1A). DATE instability occurred at a frequency of 14% (11/78 cases). Notably, we found that DATE is polymorphic in nature as 17% (13/78) of cases harbored truncated DATE variant in the tumor samples as well as in their corresponding normal tissues (Figure 1B) suggesting that the truncated DATE variant is heritable. Thus overall 24/78 (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 1A) at a frequency of 7.7% (46/592). We compared the frequency of truncated DATE variant in CRC versus normal population and found that DATE truncation significantly associates with the incidence of CRC ($P < 0.0001$, 2- tailed Fisher's Exact test). As DATE

resembles a microsatellite, its mutation in the tumor could be due to microsatellite instability (MSI) provoked by deficient 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 five mononucleotide repeat markers, NR-21, NR-24, BAT25, BAT26, MONO27 and two pentanucleotide repeats. The results revealed that nearly all of the cases with unstable DATE in the tumor exhibited the MSI high (MSI-H) phenotype, except for two cases, which were classified as microsatellite stable (MSS) according to the MSI markers but had unstable DATE (Figures 1C–E, and Supplementary Table 2) present only in the tumor and not in the adjacent normal tissue and are discussed below. 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 (IHC) for determination of MMR status. We performed IHC and the data clearly showed that all DATE unstable tumors either lack *MLH1* or *MSH2* protein, while DATE stable tumors have robust expression of both (Figure 1D). The two DATE mutant tumor cases mentioned above that were classified as MSS but showed DATE instability exhibited defective *MLH1* protein expression (Figure 1E). Next, we studied seven 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), LoVo (17A). In contrast, wild type DATE was observed in the two MSS and MMR proficient cell lines, HT-29 (30A) and SW-620 (30A) (Figure 1F and Supplementary Figures 1B, 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 which has DATE with 16A. The data revealed that these cell lines with truncated DATE also have MSI (Supplementary Table 3).

DATE truncation causes reactivation of the *HGF* promoter leading to autocrine production of HGF in CRC

Our next aim was to examine the functional consequences of DATE truncation on HGF promoter activity. These studies revealed that Shortening of DATE even by two nucleotides resulted in a significant increase in HGF promoter activity as compared to wild type DATE (30A), suggesting that DATE truncation relieves its repressor function on the *HGF* promoter (Supplementary Figure 2A–D). To determine whether DATE truncation affects endogenous HGF expression in CRC cells, we analyzed the aforementioned human CRC cell lines for HGF and Met expression. We discovered that HT-29 and SW620 cells, which have wild type DATE, do not express *HGF* mRNA and protein whereas those harboring truncated DATE (namely HCT116, HCT-15, RKO, SW-48, LOVO) do (Figure 2A–C, also see Supplementary Figure 2E). All CRC cell lines expressed abundant *Met* mRNA and protein (Figure 2A–C). Immunoblotting for Met phosphorylation showed that Met was in an activated state in CRC cells which had truncated DATE and expressed HGF (Figure 2C), thus suggesting that an autocrine HGF-Met signaling loop is operational in these cells. Next, we asked whether DATE truncation by itself was sufficient to cause activation of the endogenous HGF gene. We replaced the endogenous wild type DATE in HT-29 CRC cells with a truncated version (DATE with 12A which is the shortest DATE we have observed in

some tumor cells) by using a somatic gene targeting approach and a recombinant adeno-associated virus (rAAV) vector for gene replacement (Figure 2D, Supplementary Figure 3A, and Supplementary Table 4). We verified that the targeting event replaced the endogenous *DATE* with the truncated form by sequencing the DATE region (Supplementary Figure 3B). The results showed that HT-29 clones, harboring the truncated DATE variant exhibit activation of the *HGF* gene expression (Figure 2D).

We subsequently assessed the status of HGF and Met protein expression in DATE wild type and DATE mutant human colorectal tumor tissues. We found that tumors with mutant DATE had robust HGF expression as compared to those with wild type DATE (Figures 3A and B). HGF upregulation in the tumors also correlated with defective MMR, and Met was ubiquitously expressed at a similar level in both normal and tumor tissues (Figure 3A and Supplementary Figure 3C). Notably, cases showing truncated DATE due to polymorphism (that is, truncated DATE is present in both the tumors and matched adjacent normal tissue), expressed high HGF in the tumor and in the adjacent normal tissue at similar levels (Supplementary Figure 3D). Please note that since DATE truncation results in HGF gene activation (gain-of-function), a single truncated allele is sufficient to cause HGF expression.

DATE mutant CRC cells have operational HGF-Met signaling and are addicted to the HGF-Met signaling axis for cell growth

Our next goal was to investigate the functional consequences of aberrant HGF expression by CRC cells on their growth behavior. We hypothesized that HGF-producing CRC cells have an “oncogenic addiction” to the HGF-Met axis. To test this possibility, we performed experiments aimed at blocking HGF-Met with an HGF neutralizing antibody and also with a Met kinase inhibitor SU11274¹¹ and assessed the outcome at the cellular and molecular levels. We observed that activation of Met and its downstream signaling mediators like Akt and Erk, as well as cell growth and viability, were dampened in response to HGF neutralization and Met inhibitor (Figure 4, and Supplementary Figure 4A). Conversely, treatment of serum-starved CRC cell lines with exogenous recombinant human HGF resulted in rapid activation of Met and its downstream targets Akt and Erk and promotion of cell growth (Supplementary Figures 4B–D). Together these results revealed that DATE mutant CRC cell lines have a functional HGF-Met signaling system and depend on this axis for growth. Notably, Met inhibited cells exhibited phenotypic characteristics of cell death such as rounded morphology and detachment (Figure 4D). Assessment of markers of apoptosis such as caspase-3 and PARP cleavage showed only marginal impact (Supplementary Figures 4E and F) suggesting that inhibition of Met may cause cell death by a means other than apoptosis. One such cell death pathway that could be necroptosis. The release of High Mobility Group Box 1 protein (HMGB1) is a well-documented indicator of necroptosis¹². Thus we analyzed SU11274-treated cells for release of this marker and discovered that Met inhibition indeed resulted in a dose-dependent increase in the HMGB1 release in response to SU11274. On the other hand, HGF treatment totally abrogated HMGB1 release induced by hydrogen peroxide, a well-known inducer of cell death by necroptosis (Supplementary Figure 4G). These data indicated to us that the HGF-Met signaling axis has an anti-necroptotic function. This idea was further supported by the results of experiments in which we found that SU11274-mediated cell death could be

rescued by addition of Necrostatin-1 (Nec-1), a specific allosteric inhibitor of RIPK-1, which is a serine-threonine kinase involved in executing necroptosis¹³ (Supplementary Figure 4H). These results led us to postulate that the HGF-Met axis may modulate necroptosis through impacting RIPK-1.

HGF-Met signaling impacts RIPK-1 in CRC cells promoting escape from necroptosis

Given that RIPK-1 is the key player of cell death induction by necroptosis,^{14–16} we asked whether HGF-Met signaling has an impact on RIPK-1. We discovered that inhibition of Met by SU11274 induced a marked upregulation of RIPK-1 in CRC cell lines (Figure 5A and Supplementary Figure 5A). On the other hand, activation of Met by exogenous HGF resulted in reduction of RIPK-1 abundance, (Figure 5B and Supplementary Figures 5B, C). The downregulation of RIPK-1 was highly specific to HGF treatment and could not be observed when CRC cells were treated with staurosporine, or the agonistic anti-Fas antibody, CH11 (Supplementary Figure 5C). We next examined human CRC tissues for RIPK-1 and HGF expression and found that those with upregulated HGF expression have significantly lower levels of RIPK-1 protein when compared to those with low HGF (2-tailed unpaired t-test, $P=0.00016$; Figure 5C, Supplementary Figure 5D and Supplementary Table 5). Linear regression analysis revealed a significant ($P=0.007$) negative correlation between HGF and RIPK-1 protein levels: high HGF protein abundance significantly associated with lower levels of RIPK-1 protein (Supplementary Figure 5E). We next asked whether DATE mutagenesis in CRC influenced patient prognosis. We found that DATE mutant tumors were significantly larger as compared to DATE wild-type tumors (average tumor size of 7.27 cm vs. 4.96 cm [2-tailed unpaired t test, $P=0.003$]). High HGF and low RIPK-1 protein abundance also independently associated with advanced tumor stages (2-tailed unpaired t-test, $P=0.001$; Supplementary Figure 5F). Kaplan-Meier survival analyses indicated that patients having tumors with truncated DATE have a lower 5-year overall survival rate than those with wild type DATE (Figure 5D) (Log-rank test $P=0.006$). Further subclassification revealed that cases having MSI and truncated DATE (i.e. DATE instability) or MSS and truncated DATE (i.e. truncated DATE due to polymorphism), also exhibited a lower 5-year survival rate than those having MSS and wild type DATE (Log-rank test $P=0.014$) (see Supplementary Figure 5G) suggesting that HGF may be the influencing factor. In fact, stratification of cases based on HGF protein expression showed that those with high HGF level in the tumor have a significantly poorer survival rate as compared to those that have low HGF (Log-rank test $P=0.0001$, hazards ratio = 4.2) (Figure 5D). We also assessed patient survival rate as a function of RIPK-1 protein abundance and found that cases with low tumor RIPK-1 have poorer overall 5-year survival ($p=0.02$, hazard ratio = 2.7) as compared to those with high RIPK-1 (Figure 5D). Finally, grouping of cases based on TNM stage (i.e. stages I and II versus III and IV) uncovered that DATE truncation, HGF overexpression and RIPK-1 deficiency significantly ($P=0.02$, 0.001 and 0.05, hazards ratio = 4.0, 3.6, 2.3, respectively) reduced survival rate only in group that are at an advanced stage (stages III and IV) (Supplementary Figure 5H).

HGF-Met signaling directly impacts RIPK-1

The above data prompted us to decipher the underlying mechanisms of HGF-Met mediated RIPK-1 down regulation. It is known that RIPK-1 activity and function is regulated by

ubiquitination which occurs after it is recruited to the plasma membrane upon stimuli such as TNFR-I activation by TNF- α ^{14–16}. Therefore we performed experiments to test if Met activation by HGF causes RIPK-1 recruitment to plasma membrane and its ubiquitination. The results revealed that treatment of CRC cells with HGF causes rapid recruitment of RIPK-1 to plasma membrane (Figures 6A, B and Supplementary Figures 6A–C) and its polyubiquitination which was of Lys48-linked type (Figure 6C and Supplementary Figure 7). It should be noted that HGF-induced RIPK-1 recruitment to the plasma membrane and ubiquitination could be further stabilized when HGF treatment was performed in the presence of proteasome inhibitor MG-132 (i.e. Figures 6A and C), supporting the notion that recruited RIPK-1 is rapidly degraded by proteasome. Given the fact that Met is a tyrosine kinase and RIPK-1 could be recruited to the plasma membrane by Met activation, we asked if Met can tyrosine phosphorylate RIPK-1 hence targeting it for ubiquitination. Thus we performed immunoprecipitation and western blot studies using RIPK-1 and phosphotyrosine antibody to see if RIPK-1 becomes tyrosine phosphorylated by Met. We discovered that HGF treatment indeed induced tyrosine phosphorylation of RIPK-1 (Figure 6D). These data (Figures 6B and C) also suggested that RIPK-1 associates with Met and that it is likely become tyrosine phosphorylated by Met directly. To test this, we performed in vitro kinase assays as previously described in ¹⁷. We incubated pure recombinant active human Met kinase (His-tagged, 55 kDa) with pure recombinant active RIPK-1 (GST-tagged full-length RIPK-1, 110 kDa) in kinase buffer and assessed for RIPK-1 tyrosine phosphorylation. Incubation of Met with RIPK-1 resulted in robust tyrosine phosphorylation of RIPK-1 (Figure 6E). Proteomic analyses using MS/MS MALDI-TOF identified that RIPK-1 become tyrosine phosphorylated by Met on Tyr384 (Y384) residue. The sequence of this site is K³⁷⁷LQDEANYY³⁸⁴HL and is located in the intermediate domain of RIPK-1 near Lys377 (K377), which is the major site of RIPK-1 ubiquitinylation ^{15, 16, 18}. We found that this Tyr site and the motif (KLQDEANYHL) both are highly conserved in various organisms from humans down to at least *Xenopus*, thus indicating its functional importance. We subsequently asked whether tyrosine phosphorylation of RIPK-1 has functional consequences. It is known that RIPK-1 activates itself through the autophosphorylation of the serine residues within its kinase domain ¹⁴. Notably, we found that tyrosine phosphorylation of RIPK-1 by Met resulted in marked reduction of RIPK-1 kinase activity (i.e. RIPK-1 serine autophosphorylation) in cell-free (Figure 6E) and cell-based assays (Figure 6D).

Discussion

Development and clinical progression of sporadic and familial forms of CRC is directly linked to the presence of genomic instability ^{19–22}. Thus, gaining a better understanding of the cellular and molecular consequences of genomic instability in human CRC will aid the development of more effective treatment strategies. Here, we show that genomic instability provoked by defective DNA MMR system (which manifests as MSI) causes truncation (shortening) of an *HGF* gene promoter element which we named DATE. This promoter alteration reactivates the otherwise silenced *HGF* gene in colonic epithelial cells, leads to autocrine HGF production and dysregulated Met signaling in CRC cells. The MMR system is indispensable for maintaining fidelity of DNA replication and genomic integrity,

particularly at microsatellite regions²³. MSI is documented to play important roles in a number of other malignant conditions (beside sporadic CRC and hereditary non-polyposis colorectal cancer [HNPCC Lynch syndrome and its associated cancers]), such as cancers of the endometrium, stomach, pancreatic-biliary tract, small intestine, urothelium, ovary and brain (glioblastomas)^{21, 23–25}. We predict that in all of those cancer types with MSI, DATE is also truncated and that HGF is expressed aberrantly hence promoting tumorigenesis.

Multiple studies have reported important role for poly-dA tracts in transcription repression and shortened adenosine repeat versions are documented to disrupt histone-DNA interactions and increase transcription factor accessibility^{26–29}. Studies have shown that polydA tracts are inherently unstable and prone to hypermutability in germline cells, which will pass to progenies³⁰. Of significance, we found that the truncated DATE variant is polymorphic (that is heritable) and that the truncated variant form of DATE occurs in the normal population at an overall frequency of about 8%. We speculate that carrying a germline truncated DATE variant (hence hyperactive HGF promoter variant) may be a double-edged sword for individuals harboring it, as truncated DATE can provide robust HGF expression and promote efficient tissue regeneration and repair, but it also can instigate tumorigenesis if sufficient number of genetic/epigenetic lesions in key oncogenes and tumor suppressor genes accumulate. Future large population and prospective studies are warranted to see if individuals with truncated DATE variant are predisposed to CRC.

We documented here that DATE instability has the hallmarks of microsatellite instability (MSI) and that DATE mutation and HGF overexpression in CRC correlate with larger tumor size and poor prognosis. The importance of MSI and its influence on colon cancer patient prognosis is, however, unclear and rather contradictory. Some studies have shown no survival difference between MSI and MSS groups, whereas others have even reported a favorable outcome in the MSI group as compared to the MSS group^{22, 31}. It has been suggested that several factors influence the results obtained from different studies, such as different thresholds used to assign MSI and notably, in almost all of the published studies, MSI-low cases were bundled with the MSS group³². Obviously more biologically relevant biomarkers (like DATE and HGF) are needed to accurately stratify subjects in order to better predict prognosis and guide rational treatment. We propose that DATE alteration is not inert as it likely provides a growth advantage to cells by causing aberrant HGF expression endowing them with an autonomous growth stimulus and escape from cell death including necroptosis. This could then result in the clonal expansion of such “tumor initiating cells” (see our proposed model in Figure 7).

We suggest that DATE could be a good marker for MSI detection especially MSI-low cases, since standard MSI markers (which included five mononucleotide repeat markers such as BAT25, BAT26, BAT40, MONO27 and NR-21) failed to detect MSI in two MMR deficient CRC cases whereas DATE did (please see Figure 1E). Our findings have important clinical implications as assessing CRC samples for DATE status, HGF overexpression and also RIPK-1 down regulation could serve as robust biomarkers for stratifying CRC patients for prognosis purposes and for rational therapy targeting HGF-Met. Blocking the HGF-Met system, sensitizes CRC cells to apoptotic and necrotic stress, and most chemotherapeutic regimens (i.e. 5-FU) kill cells by these two modes of cell death. The targetable nature of

HGF-Met signaling in CRC was recently demonstrated using a selective small molecule Met kinase inhibitor, ARQ 197 (Tivantinib), which has shown significant promise in phase I trials of CRC³³. Our work encourages further exploration to develop modalities by which HGF-Met signaling can be efficiently targeted in the tumors for the treatment of CRC, especially for those subsets that harbor DATE alteration and hence aberrant HGF production. Also closer clinical surveillance and screening for CRC development in subjects who are polymorphic for truncated DATE variant (DATE mutant carriers) may be beneficial for preventing colorectal cancer burden.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

DATE instability (deletion mutagenesis) occurs in human colon carcinomas as a result of genomic instability provoked by defective DNA Mismatch Repair (MMR). (A) Representative genotyping from different human CRC cases showing DATE status in tumors (T) as compared to the corresponding adjacent normal (N) tissues. Arrows indicate DATE length. (B) Representative genotyping results demonstrating that DATE is polymorphic in nature. (C) DATE instability correlates with MSI status. The numbers one to five refer to the standard MSI markers, NR-21, BAT26, BAT25, NR-24, and MONO27, respectively. The arrows indicate the presence of instability. (D) Immunohistochemistry (IHC) revealing the absence of MLH1 or MSH2 in tumors having DATE alteration. (E)

DATE could be a sensitive indicator of MSI. IHC documenting that tumor with unstable DATE is indeed MMR deficient (despite having no apparent alterations of the standard MSI markers), as it lacks MLH1. (F) Human CRC cell lines with known MMR deficiency also have unstable DATE.

Figure 2.

DATE truncation causes *HGF* promoter activation leading to autocrine HGF production. (A–C) Human CRC cell lines with truncated DATE express *HGF* gene and protein as determined by RT-PCR (A), immunofluorescence (B) and western blotting (C). In C, pMet is phosphorylated Met using pTyr 1234/1235 antibody. (D) Replacement of endogenous wild type (30A) DATE in HT-29 cells with the truncated version (12A) using somatic gene targeting. RT-PCR indicates the presence of *HGF* transcript in the targeted clones that incorporated the mutant DATE construct. cDNA amplified from the DATE mutant cervical carcinoma cell line, C33A, was analyzed in parallel as a positive control.

Figure 3. DATE truncation correlates with HGF up-regulation in human CRC tissues. (A) Representative IHC for HGF, MET and the MMR proteins, MLH1 and MSH2, in DATE wild type and DATE unstable human CRC (*top panel*) and quantification of HGF protein abundance using Image Analysis Software (*bottom panel*). (B) Quantification of HGF abundance by western blot (*top panel*) and densitometry (*bottom panel*).

Figure 4.

The HGF-Met signaling axis is operational in CRC cells. (A) Western blot results showing that HGF neutralization inhibit Met activation and its downstream effectors in DATE mutant CRC cells. (B) Representative western blot showing efficient inhibition of Met by SU11274. (C, D) Met inhibition affects growth of CRC cell lines especially in those with mutant DATE as determined by MTT assay (C). Representative phase contrast microscopy images of CRC cells treated with SU11274 for 24 hours (D).

Figure 5.

HGF-Met signaling down regulates RIPK-1 in CRC cells. (A) Western blots indicating Met inhibition causes upregulation of RIPK-1. (B) Conversely, Met activation by HGF treatment down regulates RIPK-1. (C) Representative IHC for HGF and RIPK-1 (*top panel*) and its quantification (*bottom panel*). (D) DATE truncation, HGF upregulation as well as RIPK-1 down regulation independently associate with poor overall patient survival as determined by Kaplan Meier analyses. Log-rank test, $p=0.0057$; 0.0001 ; and 0.02 , for DATE, HGF, and RIPK-1, respectively.

Figure 6.

HGF-Met signaling impacts RIPK-1. (A) HGF stimulates association of RIPK-1 with the plasma membrane. CRC cells were treated with the indicated reagents and time (15 and 30 minutes) and analyzed for RIPK-1 by western blot (C denotes cytosolic and M membrane fractions). Actin is shown for the 30-minute time point. (B) Immunofluorescence microscopy images documenting HGF induces RIPK-1 association with the plasma membrane within 30 minutes using RIPK-1 and Met antibodies. (C) HGF induces RIPK-1 polyubiquitination. SW-620 cells were treated with or without HGF in the presence of absence of the proteasomal inhibitor MG-132 and their lysates were subjected to IP with RIPK-1 antibody and then western blotting using the indicated antibodies. (D) Samples in (C) were examined for RIPK-1 phosphorylation status using phosphotyrosine and phosphoserine antibodies. (E) Met directly phosphorylates RIPK-1. Pure recombinant human Met Kinase was incubated with pure recombinant human RIPK-1 in kinase buffer for 30 minutes and western blotting as in (D). Bargraphs depict quantification of RIPK-1 activity (RIPK-1 autophosphorylation) data from (E). Please see Materials and Methods for more details.

Figure 7.

A model for causes and consequences of HGF gene mutation in human CRC cells.