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GLOBAL HYPOMETHYLATION OF GENOMIC DNA IN CANCER-ASSOCIATED MYOFIBROBLASTS

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Abstract

Global loss of methylation has long been recognized as a feature of the malignant epithelial component in human carcinomas. Here we show evidence for this same type of epigenetic alteration in cancer-associated stromal myofibroblasts. We used methylation-sensitive SNP array analysis (MSNP) to profile DNA methylation in early passage cultures of stromal myofibroblasts isolated from human gastric cancers. The MSNP data indicated widespread loss of methylation in these cells, with rare focal gains of methylation, conclusions that were independently validated by bisulfite sequencing and by a methylation-sensitive cytosine incorporation assay. Immunohistochemistry (IHC) using anti-methylcytosine (anti-methyl-C) in a series of gastrectomy specimens showed frequent loss of methylation in nuclei of both the malignant epithelial cells and the alpha smooth muscle actin (ASMA)-positive stromal myofibroblasts of both intestinal type and diffuse carcinomas. We confirmed this phenomenon and established its onset at the stage of non-invasive dysplastic lesions by IHC for anti-methyl-C in a transgenic mouse model of multi-stage gastric carcinogenesis. These findings indicate similar general classes of epigenetic alterations in carcinoma cells and their accompanying reactive stromal cells and add to accumulating evidence for biological differences between normal and cancer-associated myofibroblasts.

INTRODUCTION

Stromal cells are increasingly recognized as influencing the biological behavior of human cancers. Among the various stromal cell types much recent research has focused on cancer-associated fibroblasts, which often acquire a myofibroblast phenotype, defined by strong expression of alpha smooth muscle actin (ASMA). The presence of such reactive cells originally suggested the classical concept of cancer as a non-healing wound (1), and a number of observations, including clinicopathological correlations and functional studies, have more recently implicated these cells as actively contributing to cancer growth, invasion and

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metastasis (2–6). In parallel with this work several laboratories have recently extended the fields of cancer genetics and epigenetics to examination of stromal cells, and there is now evidence, albeit in part controversial, for point mutations, loss of heterozygosity (LOH), and both losses and gains of DNA methylation at specific loci in cancer-associated stroma (7–15). Here we describe global loss of DNA methylation and focal gain of DNA methylation, without gross chromosomal instability, in stromal myofibroblasts of gastric carcinomas.

MATERIALS AND METHODS

Cells and Tissues

Tissue from human gastrectomy specimens was obtained immediately after resection from the Department of Surgery, University of Szeged, Szeged, Hungary. The study was approved by that institution's Ethical Committee (2069/2006). Materials were taken from the cancer and from macroscopically normal gastric corpus mucosa resected at least 5 cm from the tumour margin. Both normal and cancer samples were confirmed by histopathology. Samples from the resected tissues were placed in ice cold calcium and magnesium free Hanks balanced salt solution (HBSS) within 5 minutes. Myofibroblasts were prepared as reported previously (16). Briefly, gastrectomy tissues were transferred on ice immediately to the laboratory. The tissues were washed multiple times in calcium and magnesium free HBSS and were afterwards incubated with 1 mmol/l DTT (Sigma, St Louis, Missouri, USA) for 15 minutes followed by six sequential 30 minute incubations in 1mmol/l EDTA while gassing with 95% O₂/5% CO₂ in a shaking water bath. The primary cultures were initially maintained in RPMI 1640 containing 10% FCS which was changed to DMEM after 20–25 days of culture. The myofibroblasts were passaged a maximum of 3 to 4 times to accumulate sufficient early-passage material for these experiments. In two cases DNA was isolated from myofibroblasts both after 3 and 6 passages, to assess the effect of passage number on DNA methylation. Myofibroblasts were stained for ASMA and vimentin from each preparation to monitor purity; preparations with >1% ASMA and vimentin-negative cells were rejected. DNA was isolated using QIAamp DNA mini kit (QIAGEN, Valencia, CA). Formalin-fixed/paraffin-embedded (FFPE) and frozen sections of primary gastric carcinomas and adjacent gastric tissue as well as sections of benign gastric ulcer specimens were obtained from the Molecular Pathology Shared Resource of the Herbert Irving Comprehensive Cancer Center at Columbia University. All tissues were obtained as anonymous specimens, with the pathological diagnosis but without patient identifiers. Formalin-fixed/paraffin-embedded (FFPE) tissue was also obtained from IL1- β transgenic mice. In these animals interleukin 1-beta (IL-1 β) cDNA is expressed from the murine H/K-ATPase promoter (17). These animals develop gastric epithelial dysplasia 6–9 months after infection and 30% of the male mice develop carcinoma-in-situ in the subsequent 6–8 months (manuscript in preparation).

MSNP Probes

The preparation of MSNP probes was essentially as previously described (18,19). Genomic DNA samples, 250 ng, were first digested in the appropriate low-salt buffer with 10 units of *HpaII* or with *MspI* or mock-digested (no enzyme) in a volume of 12 microliters for 3 hours. The buffer was then adjusted to higher salt concentration by adding 3.2 microliters of 10X buffer 3 (New England Biolabs, Ipswich, MA) and 10 units of *StyI* restriction enzyme was added, for a further 3 hours of digestion. The enzymes were heat-inactivated at 65 degrees for 20 minutes and the digested DNA samples (*StyI* alone, preparation in duplicate; *StyI*+*HpaII*, preparation in duplicate; *StyI*+*MspI*, single preparation; for a total of 5 probe preparations per original DNA sample) were brought forward for linker ligation, amplification by PCR with linker primers, fragmentation and labeling with biotin as specified in the Affymetrix protocol. The resulting probes were hybridized to 250K *StyI* SNP arrays (5 arrays per biological sample), washed and scanned according to the Affymetrix protocol.

MSNP Data Analysis

The MSNP data (.cel files) were analyzed in dChip ((20); <http://biosun1.harvard.edu/complab/dchip/>) by normalization, model-based expression (perfect match/mismatch and perfect match-only methods gave similar results in this dataset), and chromosome analysis. This sequential procedure in dChip is identical to that which is typically used for assessing DNA copy number. We first analyzed the *StyI*-only array data from the myofibroblasts samples for possible changes in DNA copy number, loading these files together with SNP array data from 4 normal peripheral blood controls, and after finding no detectable chromosomal or subchromosomal gains or losses, in the sample information file we next assigned a numerical ploidy of “2” to all of the *StyI*-only arrays from the myofibroblasts, leaving the numerical ploidy field blank for the *StyI+HpaII* and *StyI+MspI* arrays. This strategy allowed us to visualize, using the chromosome view in dChip, the methylation status of *HpaII* sites flanking a given SNP-tagged locus as reduced signal intensity in the *StyI+HpaII* representations, compared to the *StyI*-only representations. The *StyI+MspI* representations allowed us to determine the reliability of the Class 2 SNPs, with greatest reliability indicated by the strongest and most consistent reduction in signal intensity in the *StyI+MspI* representations, compared to the *StyI*-only representations. To quantitate the degree of methylation at each Class 2 SNP we derived a methylation index by carrying out normalization and model-based expression in dChip, exporting the resulting SNP intensity values, subtracting the average *StyI+MspI* value at each SNP as background, and then calculating the percent preservation of intensity in *StyI+HpaII* compared with *StyI*-only.

Bisulfite Sequencing

Genomic DNA, 1 microgram, was bisulfite-converted using the CpGenome universal DNA modification kit (Millipore, Billerica, Massachusetts), according to the instructions of the manufacturer. Sequences including or adjacent to the index SNPs were amplified by PCR, using primers designed with the MethPrimer program (21) (<http://www.urogene.org/methprimer/>). PCR conditions, primer sequences, and corresponding unconverted genomic sequences were: SNP_A-2105245 (rs10259620; HOXA9) bis-For TGAGAGTGGTTTTTTTATTGTTATTG; bis-Rev CCCCAATTTTACCTCTAACTAACTTT (unconverted sequences For TGAGAGTGGTCTTTCCACTGCTACTG; Rev CCCCAATTTTGCCTCTGGCTGGCTTT); SNP_A-2034029 (rs11079830, HOXB6) bis-For, TTTAGGTGTGGTGTTTAAAGAATGT; bis-Rev, CAAATTAAATTTCCCCAAAAAAA (unconverted sequences For, CCTAGGTGTGGTGTCCAAAAGAATGC; Rev, CAGATTGGGGTTTCCCCAAAAGA). The PCR products were cloned using the TopoTA Cloning System (Invitrogen) and the resulting plasmids sequenced.

³H-dCTP Incorporation Assay

The cytosine extension assay was performed using 0.5 µg DNA as previously described (22). A standard curve was established using fixed ratios of universal methylated and unmethylated DNA (Millipore, Billerica, MA). DNA was digested with 20 U of *HpaII* for 18 hours at 37°C. The cytosine extension reaction was performed by adding 3 µl of 10× PCR Buffer II, 0.8 µl of MgCl₂, 0.75 U of AmpliTaq DNA polymerase (Applied Biosystems, Branchburg, NJ) and 0.15 µl of ³H-dCTP (57.4 Ci/mmol)(PerkinElmer, Boston, MA) to the digested product and incubated at 56°C for one hour. Samples were applied to DE-81 ion-exchange filters and washed three times with 0.5M Na-phosphate buffer. After air-drying the filters were processed for scintillation counting. The ³H-dCTP incorporation into DNA was expressed as mean disintegration per minute (DPM) per µg of DNA. Assuming a linear relationship between DPM

and % methylation (22), the degree of methylation in the samples was estimated based on measurements of fixed ratios of control methylated and unmethylated DNA.

Antibodies and Immunohistochemistry

For detecting nuclear methyl-C in FFPE and frozen tissue sections we used a mouse monoclonal antibody, 5-methylcytosine (Ab-1) (Calbiochem, San Diego, CA) which has been previously validated for this purpose (23,24). Antigen retrieval in FFPE sections was by microwave heating for 7 minutes at the high setting (bringing the solution to a boil) followed by 7 minutes at the low setting (during which the solution was maintained at a gentle boil) in TBS-T (1× TBS with 0.1% Tween 20) containing 1M sodium-citrate. After Ag retrieval the FFPE sections were exposed to 3.5 N HCl for 15 minutes at room temperature and washed in TBS-T. Subsequently sections were treated with 0.3% peroxidase to quench endogenous peroxidase activity and were incubated with 5% normal hoarse serum (NHS) for 30 minutes followed by overnight incubation with the anti-methyl-C monoclonal antibody at a 1:1000 dilution at room temperature. The majority of slides contained both cancerous and normal tissue but when such a slide was not available both control and cancer slides were treated identically. For detection of ASMA and DNMT (both antibodies from Abcam, Cambridge, MA) the same antigen retrieval procedure was employed. Slides were not exposed to HCl and a rabbit polyclonal antibody was used at 1:100 dilution. Antibody detection was performed by incubation with biotinylated goat anti-mouse and anti-rabbit secondary antibodies (Dako, Carpinteria, CA) at a 1:200 dilution for 30 minutes at room temperature. Slides were developed for 30 seconds to one minute using the DAB staining kit (Dako, Carpinteria, CA) and were counterstained with hematoxylin. For dual staining of methyl-C and ASMA the above procedure was followed for the anti-methyl-C staining and subsequently slides were incubated with the ASMA antibody and developed using the BCIP/NBT alkaline-phosphatase substrate kit (Vector Labs, Burlingame, CA). Frozen sections were fixed in acetone and were not subjected to antigen retrieval. Sections were exposed to 3 N HCl for ten minutes at room temperature and washed in PBS. Simultaneous incubations with both primary antibodies at 1:1000 dilution for anti-methyl-C and 1:100 dilution anti- α SMA were overnight at 4°C in PBS containing 5% BSA and 3% NHS. Subsequently secondary Abs (Texas Red labeled donkey anti-goat and FITC labeled donkey anti-rabbit) at 1:300 dilution was used for detection (Jackson Immuno, West Grove, PA). The intensity of nuclear staining was measured using the ImageJ software (Image J v 1.38, Millersville, PA) after outlining the nuclear area of ASMA-positive intraepithelial cells.

Quantitative real time RT-PCR

Quantitative real time RT-PCR was performed using a 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions were performed in triplicate in 96-well optical reaction plates. Each reaction contained cDNA reverse transcribed from 5 ng total RNA, 1X Power SYBR Green PCR master mix (Applied Biosystems Inc.) and 0.2 μ M of each specific primer pair, which were designed using online D-Lux software (Invitrogen) or Primer Express 3.0 Software (Applied Biosystems) with at least one primer spanning two exons (*DNMT1*, F: TTCAAATTCTGTGTGAGCTGTGC, R: cgggtGTGGCTGAGTAGAGGACCcG; *DNMT3a*, F: ATTGATGAGCGCACAAGAGAGC, R: cggaaGCAGATGTCCTCAATGTTCCg; *DNMT3b*, F: AATGTTGTAGCCATGAAGGTTGG, R: cgggtGGATTACACTCCAGGAACcG; *HPRT1*, TTATGGACAGGACTGAACGTCTTG, R: CCAGCAGGTCAGCAAAGAATT). The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15s at 95°C for denaturation and 1 min at 60°C for annealing and extension. The relative expression level of a target gene in a particular sample is calculated as: $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T(\text{sample})} - \Delta C_{T(\text{calibrator})}$ (23).

RESULTS

MSNP Analysis of DNA from Early-Passage Cancer-Associated Myofibroblasts

Using the MSNP procedure we analyzed genomic DNA from early passage (p3–4) myofibroblast cultures prepared from gastrectomy specimens from 2 patients with intestinal type gastric carcinomas. For each patient we compared the pattern of DNA methylation in myofibroblasts isolated from within the tumor (cancer-associated myofibroblasts) to the pattern of methylation in myofibroblasts isolated from gastric tissue of the same specimen 10 cm away from the tumor at the negative surgical margin (control myofibroblasts). As most patients with gastric cancers have underlying abnormalities of chronic gastritis, the tumor-distant myofibroblasts cannot be considered completely normal. Nonetheless, these cells from the same organ of the same individual are the closest matched control for this type of experiment, in which we sought to uncover epigenetic abnormalities in the tumor stroma. The MSNP data were from duplicate technical replicates with *StyI*-alone (*S*) and *StyI*+*HpaII* (*SH*) and a single determination with *StyI*+*MspI* (*SM*) for each of the DNA samples.

First, using the MSNP data from the *S* genomic representations, we asked whether there were detectable abnormalities of DNA copy number specific to the tumor-associated myofibroblasts. Setting the numerical ploidy as 2 in the tumor-distant myofibroblasts and processing the data by the standard approach of normalization, model-based expression and chromosome analysis in dChip, no chromosomal or sub-chromosomal aneuploidies were seen in the tumor-associated fibroblasts. To examine DNA methylation, we next compared the signal intensities in the *S* vs. *SH* genomic representations at informative SNP-tagged loci (Class 2 SNPs; (18)), bioinformatically defined as those SNPs on the array that are within *StyI* restriction fragments that contain internal *HpaII* sites. To select the most technically reliable markers, for a Class 2 SNP-tagged locus to be brought forward in the analysis we additionally required that the average intensity in *SM* was reduced by >70% compared to the average with *S*. To facilitate a numerical analysis for losses and gains of DNA methylation we next calculated a methylation index (MI) for each Class 2 *StyI* fragment, first subtracting the mean value for the *SM* representations as background, and then using the formula $(SH_{av}-S_{av})/S_{av}+1$, which yields a value near zero for an unmethylated locus and a value near 1 for a methylated locus. We imported the resulting values to dChip and displayed these MI values by chromosome position. As shown visually and numerically in Figure 1a and b, this procedure indicated that in both patients the tumor-associated myofibroblast DNA was hypomethylated on average at *HpaII* sites adjacent to the Class 2 SNPs queried by these arrays, compared to the DNA isolated from the control myofibroblasts distant from the tumors. This loss of methylation was more severe in Case 45, but the phenomenon was also seen in Case 42.

Validation and Extension of the MSNP Data by Bisulfite Sequencing and a [³H]dCTP-Incorporation Assay

To search for loci with recurrent loss of methylation, and to ask whether any loci queried by the array might have undergone the opposite phenomenon of gain of methylation, we first eliminated those Class 2 loci that did not vary substantially in MI across the 4 biological samples and then subjected the MI values for the remaining Class 2 loci to supervised hierarchical clustering. This procedure identified a substantial group of loci with consistent loss of methylation and a much smaller group with consistent gain of methylation. We chose 2 of these loci, one with loss of methylation and one with gain of methylation reported by MSNP, for independent validations using a standard procedure of bisulfite conversion followed by PCR, cloning and sequencing. This procedure scores methylation not only at *HpaII* sites but also at all CpG dinucleotides between the PCR primers. As shown in Figure 2, the bisulfite sequencing results matched the direction of the changes seen in the MSNP data, and showed that the altered methylation affected multiple CpG sites in addition to the *HpaII* sites.

To independently confirm the global loss of methylation reported by MSNP we used a cytosine extension assay that has been previously validated for assessing global genomic methyl-C (22). This assay measures the incorporation of [³H]dCTP in a fill-in reaction on CG overhangs in *HpaII* digested genomic DNA. As shown by the standard curve and experimental results in Figure 3 there was a significant decrease in methylation content in both of the cancer-associated myofibroblast samples that had been subjected to MSNP analysis, as compared to the tumor-distant myofibroblast control samples. We also performed this global methylation assay on genomic DNA from three additional cases of matched cancer-associated and control myofibroblast preparations and found a similar decrease in methyl-C content in all cases (Fig. 3b). In order to also assess the effect of prolonged passaging in culture on methyl-C content we isolated DNA from myofibroblasts comparing the results at 3 vs. 6 passages. As predicted from the prior literature (24), we observed a decrease in 5-methyl-C content in the later passage cells, but the difference between the control and cancer-associated myofibroblasts was preserved at early and late passages (Supplementary Figure S1).

Anti-5-methyl-C Immunohistochemistry Showing Loss of DNA Methylation in Stromal Myofibroblasts of Primary Gastric Carcinomas

As noted above, it is well documented that patterns of DNA methylation can change as an artifact of tissue culture and increasing cell passage (24,25). Thus although we carried out our MSNP analysis on early-passage primary cultures, it was still important to verify that the widespread loss of CpG methylation observed in these samples accurately reflected the true situation in primary gastric cancers. To analyze a large series of cases we utilized immunohistochemistry (IHC) on archival formalin-fixed paraffin-embedded (FFPE) tissues, detecting nuclear 5-methyl-C using a monoclonal antibody that has been well characterized in previous studies of global loss of DNA methylation in the malignant epithelial component of several types of carcinomas in humans (26–29). First considering the normal controls for this analysis, while the precise identity of the normal precursors of cancer-associated myofibroblasts is not yet known, we observed strong nuclear staining with anti-methyl-C in both intraepithelial ASMA-positive spindle cells not associated with blood vessels (intraepithelial myofibroblasts), and in myofibroblasts of the thin muscularis mucosae layer immediately under the normal epithelium. As previously described, we noted a loss of anti-methyl-C staining in the malignant epithelial cells of the majority of the diffuse and intestinal type gastric cancers (Figure 4 and Figure 5 and Supplementary Table 1). For the FFPE analyses we identified ASMA-positive cells both by alternating anti-ASMA and anti-methyl-C in serial sections. This parallel staining revealed an obvious loss of anti-methyl-C staining in the ASMA positive myofibroblast cells in a majority of the cancer specimens (Figure 4). Tumor infiltrating lymphocytes did not exhibit a loss of staining and hence served as internal controls (Figure 4). Qualitative scoring of the anti-methyl-C IHC in the series of 10 intestinal type and 6 diffuse type gastric cancers revealed a consistent relative loss of staining intensity in the cancer-associated myofibroblasts in every case, albeit with some variation in the extent of this loss, as listed in Supplementary Table 1.

To verify the difference in staining intensity in rigorously defined myofibroblasts we also performed dual ASMA and anti-methyl-C staining on the same histological section for several of these cases, using 2-color IHC on FFPE sections as well as dual color immunofluorescence (IF) on frozen sections. As shown by the examples in Figure 5, using dual color IHC we confirmed the reduction in nuclear staining intensity for 5-methyl-C in cells in ASMA-positive cancer-associated myofibroblasts, relative to ASMA-positive non-cancer-associated myofibroblasts in 2 cases of intestinal type and 1 cases of diffuse type gastric adenocarcinoma. As an important technical point, in these cases the use of blocks from the margin of the surgical resection allowed us to capture images of the cancer-associated and non-cancer-associated stroma from a single tissue section on a single slide. For quantitative assessment of these

findings, we utilized dual color IF to examine frozen sections of the same 2 intestinal type gastric cancer cases. As shown in Figure 5 (b, c) this procedure confirmed the loss of nuclear 5-methyl-C in the cancer-associated myofibroblasts.

To begin to address whether the changes in methylation content are cancer specific or alternatively are due to non-specific stromal cell proliferation, as can occur in various reactive conditions, we carried out IHC for nuclear 5-methyl-C in 4 partial gastrectomy specimens containing benign gastric ulcers. As shown by the example in Supplementary Figure S2, comparing reactive proliferating fibroblasts in the ulcer bed to comparable cells under the adjacent unaffected gastric mucosa, there was no change in 5-methyl-C staining of these reactive stromal cells in any of the 4 benign ulcers.

Global Loss of DNA Methylation Occurs in Stromal Myofibroblasts of Dysplastic Lesions in a Mouse Model of Gastric Carcinoma

To ask whether these findings could be extended to a well controlled animal model system, we examined DNA methylation in a newly developed transgenic mouse model of gastric cancer in which an interleukin 1-beta (IL-1 β) cDNA is expressed from the gastrin promoter (17). This model is advantageous for studying tumor progression, as low grade dysplastic gastric lesions are observed by 6–9 months and progress to carcinoma in situ over the subsequent 6–8 months. When we carried out IHC for 5-methyl-C on FFPE sections of stomachs from these mice we observed a striking loss of staining in reactive myofibroblasts already evident in the early dysplastic lesions, accompanied by a definite but somewhat less pronounced loss of methyl-C immunostaining in the dysplastic epithelial cells (Fig. 6). As in the human cases, the infiltrating leukocytes maintained nuclear anti-5-methyl-C staining and served as a positive control for the IHC procedure. At the more advanced stage of in situ carcinoma both the malignant epithelial cells and the stromal myofibroblasts showed loss of nuclear 5-methyl-C (Fig. 6).

Lack of Obvious Changes in DNA Methyltransferase Expression in Cancer-Associated Myofibroblasts

DNMT1, 3a and 3b are the DNA methyltransferases responsible for methylating cytosine in CpG dinucleotides. By Q-PCR we found no evidence for strongly altered expression of the mRNAs for these enzymes between the cultured cancer-associated and control myofibroblasts (Supplementary Fig. S3). In our hands the sensitivity of IHC for detecting DNMT1 was not high enough to determine whether the amounts of this protein varied between the normal and cancer-associated myofibroblasts, as neither of these cells were detectably stained, even though the malignant epithelial cells of the gastric carcinomas were often found to be strongly positive (Supplementary Fig. S3). Additional analysis with more sensitive antibody reagents, when available, will be necessary to definitively answer whether decreased expression of DNMTs plays a role in the global reduction of CpG methylation in cancer-associated myofibroblasts.

DISCUSSION

Given the increasing evidence for a functional contribution of stromal myofibroblasts in the progression of human carcinomas, it is important to understand the genomic changes that accompany the conversion of normal myofibroblasts into cancer-associated myofibroblasts. Myofibroblasts are prominent elements of the tumor stroma in most types of human carcinomas, along with tumor-associated blood vessels and inflammatory cells. Their exact origin is an area of active research and candidate precursor cells include fibroblasts, smooth muscle cells and bone marrow derived stem cells (4). In the intestine, for example, myofibroblasts share several properties with the smooth muscle cells of the muscularis mucosae and histologically appear to arise from that layer (30), and in the invasive gastric carcinomas (intestinal type) that we have examined the histology also suggests that at least

some of these cells have proliferated from this layer. In this study we have defined myofibroblasts as spindle-shaped cells in the mucosa and muscularis mucosae that express α SMA and are not part of blood vessel walls. Smooth muscle cells of the muscularis propria are also SMA-positive, but here we have specifically dealt with areas of the gastric cancers that had not invaded this deeper layer. Intraepithelial myofibroblasts are rare but detectable in normal non-inflamed mucosa (16) and we have used both types of ASMA-positive cells (muscularis mucosae and intraepithelial) for comparing to cancer-associated myofibroblasts. It has been shown that their number (both the cellular thickness of the muscularis mucosae and the intraepithelial component) increases in pre-neoplastic tissue and in advanced cancer and that these cells come to constitute a significant portion of the tumor volume (31). Relevant to the controls in our study, myofibroblasts were originally described in non malignant wound healing and as such are also seen in gastric ulcers.

In a research area that is still evolving, recent studies have suggested that cancer-associated stromal cells undergo specific genetic and epigenetic changes. Our data shown here, from multiple modalities including MSNP for genomic methylation profiling, bisulfite sequencing, an in vitro radiolabeled cytosine incorporation assay, and anti-5-methyl-C IHC on primary gastric cancers and dysplasias, together indicate that a major phenotypic change in cancer-associated myofibroblasts is a global reduction in DNA methylation. This phenomenon parallels the overall loss of DNA methylation that has been well documented in the malignant epithelial component of multiple types of human carcinomas (32–35), including gastric carcinomas (data shown here, and prior studies, for example (36–38)). However, the cause of genomic demethylation in cancer cells remains unresolved, and likewise additional research will be needed to understand the mechanisms underlying this phenomenon in stromal myofibroblasts. Our data suggest that reduced expression of DNMTs probably does not explain this loss of methylation. Alternatively it has been suggested that a deficiency in tissue or circulating methyl donors may contribute to demethylation in cancer.

A number of hypotheses have also been put forward concerning the downstream consequences of loss of CpG methylation in neoplastic epithelial cells, including effects on gene expression and genome stability. Our MSNP data for the 2 cases of short term primary cultures of myofibroblasts isolated from within gastric cancers to cultures of myofibroblasts isolated from histologically uninvolved gastric mucosa distant from the cancers showed no evidence for genomic instability in terms of altered chromosomal or sub-chromosomal copy number, and no evidence for loss of heterozygosity (LOH). It remains to be determined whether the cause and the consequence of global hypomethylation are distinct between the epithelial or neoplastic cell population and the cancer associated stroma cells.

Emerging evidence suggests that expression of cytokines, proteolytic enzymes and their endogenous inhibitors (MMPs and TIMPs), and growth factors, are essential for the cross talk between cancer-associated myofibroblasts and malignant epithelial cells, including in gastric cancers (16,39,40). Several of these genes are up-regulated in cancer-associated myofibroblasts during the phenotypic change that accompanies a transformation from normal resting fibroblasts. Based on experiments in tissue culture with demethylating drugs, some of these genes have been suggested to be potentially up-regulated via hypomethylation (41). In general support of this observation is the finding that different patterns of gene expression are observed between cultured cancer-associated and non-cancer-associated myofibroblasts (30). In considering the functional correlates of global loss of methylation in these stromal cells, it is interesting that a recent study in a different organ system has found that decitabine treatment (inhibition of DNA methyltransferases) inhibits myofibroblast transdifferentiation from hepatic stellate cells (42). One of the most intriguing aspects of global hypomethylation in gastric cancer-associated myofibroblasts, shown by our analysis in the transgenic mouse

model, is its occurrence early in cancer progression – at the dysplastic pre-invasive stage. Thus, scoring this phenomenon by IHC may prove useful in diagnosis of early gastric lesions.

Mechanistically, loss of DNA methylation may have several causes. For example, it could be a consequence of abnormal cellular proliferation, a local deficiency of methyl donors in dysplastic gastric mucosa, or both. Early studies have suggested that certain premalignant conditions may lead to decreased tissue folate content and thus a local deficiency in methyl donors (43). This same question has persisted for many years in the context of the parallel loss of methylation seen in malignant epithelial cells, and the answer may have ramifications for chemopreventive and therapeutic strategies that deliberately seek to increase or decrease cellular DNA methylation. Supplementation with methyl donors will need to be done taking into account the balance between reversing hypomethylation and potentially increasing tumor suppressor gene hypermethylation, so it will be important to define the time course of hypo- and hypermethylation in cancer initiation and progression, which may allow a more targeted approach to chemoprevention. If, as suggested previously (44), genomic hypomethylation is an early event that precedes at least some instances of gene specific hypermethylation, the optimal timing of methyl supplementation (i.e. folate, choline, betaine, selenomethionine and S-adenosylmethionine) may be different from the optimal timing for administering methyltransferase inhibitors (i.e. EGCG, decitabine). This will be a challenging problem, as pathological gain of DNA methylation can occur very early in the tumorigenic pathway, sometimes as early as fetal organogenesis (35,45,46). More optimistically, our findings of reduced genomic methylation in cancer-associated stromal cells raise the possibility that demethylating drugs like decitabine might be able to exert anti-cancer activity in solid tumors not only via effects on the neoplastic cells, but also possibly by provoking a hypomethylation crisis in the supporting stromal myofibroblasts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MSNP data showing loss of DNA methylation in cancer-associated myofibroblasts

A, Methylation index values for Class 2 SNPs (Affymetrix 250K StyI arrays) displayed by physical position along the human chromosomes (left panel) and zoomed-in to chromosome 10 (right panel). In each of the 2 cases examined (Case 45, Case 42) the MSNP analysis was a comparison of early passage myofibroblasts isolated from within the polypoid intestinal type carcinoma (CAF) to myofibroblasts isolated from gastric tissue 10 cm away from the carcinoma (NL). Loss of methylation (darker blue color) is seen uniformly across all chromosomes, and the zoomed-in region is simply one example. **B**, Histograms of methylation index for Class 2 SNPs in the 2 cases. Both cases show a shift in the distribution to lower values in the cancer-associated myofibroblasts, with this loss being most severe in Case 45. The vertical lines indicate the median methylation index for each sample.

Figure 2. Validation of the MSNP data by bisulfite sequencing

A, Bisulfite sequencing of one of the many SNP-tagged loci that showed a reduced methylation index in cancer-associated myofibroblasts from both cases: SNP ID rs10259620; in *HOXA9* exon 2. Genomic DNA was bisulfite-treated and then amplified by PCR using primers specific for the converted sequence. The bisulfite PCR products were cloned and multiple clones sequenced (rows). There are a larger number of unmethylated CpG dinucleotides (white circles) in the cancer-associated myofibroblasts (CAF), compared to the myofibroblasts distant from the tumors (NL). **B**, Results for one of the few SNP-tagged loci (SNP ID rs11079830, *HOXB6* proximal promoter region) that showed an increased methylation index in both cases. There are a larger number of methylated CpG dinucleotides (black circles) in the cancer-associated myofibroblasts. The CpG dinucleotides in *HpaII* sites between the PCR primers are indicated by the vertical arrows. Symbols in the restriction maps are: S, *StyI*; H, *HpaII*; horizontal arrows, bisulfite PCR primers.

Figure 3. Validation of loss of DNA methylation in cancer-associated myofibroblasts by a $^3\text{HdCTP}$ -Incorporation assay

A, Calibration data from a mixing experiment with in vitro methylated and unmethylated human DNA validating the ability of this assay to quantitate the average CpG methylation of genomic *HpaII* sites. **B**, Results with genomic DNA from the early passage gastric myofibroblasts in 5 cases (#42 and #45 are cases represented in the MSNP data while the others are additional cases). There is consistent loss of methylation in the cancer-associated myofibroblasts (CAF) compared to myofibroblasts from non-cancerous gastric mucosa (NL) in all cases, albeit with some variability in the extent of loss from case to case. Mean values and standard deviations of triplicate determinations are shown.

Figure 4. Anti-5-methyl-C IHC showing loss of CpG methylation in myofibroblasts of primary gastric carcinomas

The upper and lower panels show IHC with anti-ASMA and anti-5-methyl-C, respectively. The control non-neoplastic gastric mucosa (Normal) was sampled at a location in the gastrectomy distant from an intestinal type carcinoma. The ASMA-positive spindle-shaped myofibroblasts of the normal muscularis mucosae show strong nuclear staining with anti-5-methyl-C (large arrow), as do the normal epithelial cells (small arrow) and leukocytes (lymphocytes and polymorphonuclear cells; asterisk). The more rare ASMA-positive intraepithelial myofibroblasts also showed uniformly strongly stained in multiple sections examined (example; arrowhead). Sections from within the cancers in 2 cases of intestinal type gastric carcinomas (CA; Case 1 and Case 2) show myofibroblasts (large arrows) and malignant epithelial cells (small arrows) with reduced IHC intensity of 5-methyl-C. As in internal control, the anti-5-methyl-C staining of infiltrating leukocytes remains strong in the neoplastic tissue (asterisks).

Figure 5. Dual staining to detect nuclear anti-5-methyl-C in ASMA positive cells

A, Dual color IHC (brown DAB: anti-5-methyl-C; blue NBT: anti-ASMA) showing normal and tumor areas from cases of intestinal type (left panels) and diffuse type (right panels) gastric carcinoma, with the mucosa at the uninvolved margin illustrated in the top panels (NL) and the cancerous tissue in the bottom panels (CA). There is an obvious reduction in nuclear 5-methyl-C immunostaining in the cancer-associated myofibroblasts (arrows), while the inflammatory cells surrounding the myofibroblasts have persistently high staining intensity (asterisk). **B**, Dual color IF from another case of intestinal type gastric cancer. The left panel (NL) shows a confocal scanning microscopy image (100X objective) of non-neoplastic gastric mucosa (from negative surgical margin) showing an intraepithelial ASMA+ (green) cell with strong anti-5-mc staining (red) in the nucleus (arrow). The right panel (CAF) shows a group of intratumoral ASMA+ cells in the intestinal type gastric cancer from the same patient showing decreased nuclear anti-5-methyl-C staining (arrows). **C**, Quantitation of anti-5-methyl-C signal intensity from 10 individual ASMA+ cells in two cases of gastric cancer and adjacent normal tissue. In the intratumoral myofibroblasts from both cases there is a global loss of DNA methylation.

Figure 6. Change in anti-5-methyl-C staining of myofibroblasts in an IL1 β transgenic mouse model of progressive gastric dysplasia

Strong anti-5-methyl-C staining is seen for the epithelial cells (small arrows) and ASMA positive myofibroblasts (large arrows) in normal histology IL1 β -Tg gastric mucosa. There is an obvious decrease in anti-5-methyl-C staining in both epithelial and ASMA positive cells in the dysplastic gastric mucosa, and a persistent decrease in anti-5-methyl-C staining in epithelial and ASMA positive cells in the carcinoma in situ. As an internal control, there is preserved anti-5-methyl-C staining of infiltrating lymphocytes in both the dysplasia and carcinoma in situ. These IHC findings were consistent in each of two animals examined from each of the 3 categories (normal, dysplasia, CA in situ).