Mutation in the nm23 Gene Is Associated with Metastasis in Colorectal Cancer

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Abstract

The human nm23 gene, a candidate metastatic suppressor gene, consists of two genes, nm23-H1 and nm23-H2. The potential mutation in the nm23-H1 gene was examined in colorectal cancer using a reverse transcription polymerase chain reaction amplification followed by DNA sequencing analysis. Genomic alterations in the nm23 gene were also examined by Southern hybridization. Genetic alterations either as a deletion in the coding sequence of nm23-H1 or an allelic deletion were detected in four among eight colorectal adenocarcinomas associated with metastases in lymph nodes, lung, or liver. No alteration was observed in 12 additional colorectal cancer specimens without metastasis. These results provide first evidence for novel mutation in the nm23 gene and demonstrate a correlation between the mutation in the nm23 gene and metastasis in colorectal oncogenesis which suggests that the nm23 gene plays a role in the causation of metastasis.

Introduction

A cascade of cellular, biochemical, and genetic events are known to occur in the development and progression of tumors leading to malignancy and ultimately to metastasis. A novel gene, nm23, a candidate metastasis suppressor gene, encodes a human M, 17,000 nuclear and cytoplasmic protein containing 152 amino acids (1, 2). The gene is localized on chromosome 17q22 (3). The human nm23 gene family consists of two genes, nm23-H1 and nm23-H2, with 88% homology (4). The nm23 gene is expressed significantly higher in human breast tissues having low metastatic activity in comparison to breast tumor with high metastatic activity (5, 6). Recently, nm23-H1 amplification with overexpression in RNA has been reported in neuroblastomas (7). Interestingly, an enhanced level of nm23 expression was observed in colorectal carcinomas with no relationship between the expression level of nm23 and metastatic activity (8). Recently, examining nine informative cases of colon cancer, a somatic allelic loss of nm23 in one cancer patient and a homozygous deletion in a lymph node metastasis in another case has been reported, indicating that nm23 is involved in a significant manner in tumorigenesis and metastasis (9). No information is available about whether there is a genetic alteration other than allelic change (for example, mutation) in the remaining seven cases of colon cancer.

In an attempt to determine the potential mutation in the nm23 gene in colorectal oncogenesis and possible relation with metastasis, we amplified the entire nm23-H1 coding region of colorectal tumor and corresponding normal mucosa by reverse transcription-polymerase chain reaction followed by DNA sequencing. Additionally, the somatic allelic loss of nm23 analyzed by Southern hybridization was also examined in these tumors. A preliminary study has been reported (10).

Materials and Methods

Specimens. Surgically removed colorectal tumors and corresponding normal mucosa were obtained from the Departments of Colorectal Surgery and Surgical Pathology, The Cleveland Clinic Foundation. Metastatic cases were identified by clinical and histopathological analyses of neoplastic cells in organs such as lymph nodes, liver, lung, etc., along with the primary tumor. Normal colon mucosa specimens were provided by Dr. Claudio Fiocchi, Department of Immunology, The Cleveland Clinic Foundation. Normal peripheral blood samples were obtained from the Department of Hematology, The Cleveland Clinic Foundation. The human placenta was a gift from Dr. A. W. Stegges, Northeastern Ohio Universities College of Medicine.

Reverse Transcription-PCR Amplification. Both 0.2-0.4 g tumor and corresponding normal mucosa were frozen and pulverized to powder in liquid nitrogen. Total RNA was isolated by the guanidinium thiocyanate-CsCl method (11), and 1 µg RNA was used for reverse transcription following the instructions of the SuperScript preamplification system (GibcoBRL, Bethesda, MD). First-strand cDNA was directly used for 35 cycles of PCR amplification (12) using GeneAmp PCR reagent kit (Perkin Elmer Cetus, San Diego, CA). A pair of primers, hnm23c 5': AAGAATTCTCGGGTCGAGGCCGCCATG and hnm23c 3': GGGAATTCCTCGGGAGCGCCCGATG, based on the mRNA sequence (4), were designed to amplify the entire nm23-H1 coding sequence of human nm23 cDNA (533 base pairs). PCR cycles include denaturation for 1 min at 94°C, annealing for 1 min at 42°C, and polymerization for 3 min at 72°C with a 3-s time extension/cycle. A minimum of two independent PCR amplifications from each specimen were done to avoid the misincorporation by Taq polymerase. The PCR product was separated on 3.5% NuSieve GTG low melting agarose gel and purified by the GeneClean procedure (BIO 101, San Diego, CA). The PCR products digested with EcoRI (Boehringer Mannheim, Indianapolis, IN) were repurified by GeneClean and cloned into the BlueScript KS vector (Stratagene, San Diego, CA) as described earlier (13). Plasmids with the nm23 insert were isolated from transformed XL-1 cells (Stratagene) by alkaline miniprep as described (11) and sequenced by the dideoxy method (14), using a Sequenase 2.0 sequencing kit (USB, Cleveland, OH) and [α-32P]dATP (Amersham, Arlington Hts., IL). A minimum of five individual clones from each PCR were analyzed to confirm the sequence. Southern Blot Analysis. Genomic DNA from colorectal tumors and corresponding normal mucosa was isolated as described (13). Twenty µg of DNA were completely digested with BglII and separated on 0.8% agarose gel and then transferred to Nitran membrane (13). The blots were hybridized with an α-32P-labeled 533-base pair nm23-H1 probe (entire coding sequence of normal colon mucosa produced by PCR amplification) in standard conditions (13).

Results

Reverse Transcription-PCR Amplification of nm23-H1 mRNA. Among 20 specimens examined, 13 pairs of RNA from both tumor and corresponding normal mucosa were successfully reverse transcribed; their coding sequences were amplified by PCR using a pair of primers flanking the entire coding sequence of nm23-H1. One distinct 533-base pair PCR product was detected in normal mucosa and tumors as expected, except in tumors 3 and 10. Fig. 1 shows PCR amplified products obtained from tumors 3 and 10, which showed a different pattern. A relatively smaller band of approximately 470-base pair from tumor 10 was amplified along with the 533-base pair normal...
40% of them have only a 7.6-kilobase allele and 8.5% have only a due to an early in-frame stop codon. The smaller band amplified from compared to normal corresponding tissue (Fig. 4B). Among others, 25% of patients' DNA have both 2.3- and 7.6-kilobase bands (alleles); the nm23 gene was found to be reduced markedly in tumor 7 when significantly lost in tumor 1 in comparison to its corresponding normal tissue. The 2.3-kilobase allele was significantly reduced in tumor 1 compared with its corresponding nontumorigenic tissue. There was no change in tumor 10, although this tumor carries a 64-base pair deleted coding sequence. We also noted an inverse relationship in the peak area between the 2.3- and 7.6-kilobase alleles; i.e., a reduction in the 2.3-kilobase intensity of the signal was accompanied by an amplification in the signal in the 7.6-kilobase band (tumor 1); with tumor 7, the opposite occurs. This type of DNA amplification (7.6-kilobase allele in tumor 1 or 2.3-kilobase allele in tumor 7) may be due to a rearrangement within a specific allele for the loss in the other affected allele of the nm23 gene.

**DNA Sequencing of Cloned cDNA of nm23-H1 in Normal Mucosa and Tumors.** Nucleotide sequence analyses of cloned PCR products showed that all normal size bands (533-base pair), whether amplified from normal mucosa or tumors, have the identical nm23-H1 coding sequences, which perfectly matched the published data (4). By sequence analysis of the smaller band from tumor 10, the PCR product revealed a 64-base pair deletion. Fig. 2 represents this analysis: the right panel demonstrates the deletion in this tumor while the left panel shows the normal nm23 sequence pattern in the corresponding normal tissue. The deletion has started from amino acid 86, as shown in the schematic representation in Fig. 3. The resultant deletion shifted the reading frame and changed all amino acids from 87, producing a smaller nm23 protein (122 amino acids instead of 152 amino acids) due to an early in-frame stop codon. The smaller band amplified from tumor 3 showed a totally unrelated sequence to nm23. We conducted a gene bank search for any possible sequence homology of >28%, but we found none.

**Allelic Deletion.** Southern blot analyses of 15 pairs of specimens revealed BglII RFLP as reported (3, 9). An allelic loss was detected in two tumors, as shown in Fig. 4. The 2.3-kilobase allele was significantly lost in tumor 1 in comparison to its corresponding normal mucosa (Fig. 4A). On the other hand, the 7.6-kilobase allele of the nm23 gene was found to be reduced markedly in tumor 7 when compared to normal corresponding tissue (Fig. 4B). Among others, 25% of patients' DNA have both 2.3- and 7.6-kilobase bands (alleles); 40% of them have only a 7.6-kilobase allele and 8.5% have only a 2.3-kilobase allele. A 21-kilobase band was also identified in all the DNA samples isolated from normal mucosa, tumors, placenta, and blood samples. DNA from normal human placenta and white blood cells revealed a 33% 7.6-kilobase allele and 67% of 2.3-kilobase allele in BglII RFLP (data not presented).

Comparing the densitometric analysis by a Bio-Rad video densitometer model 620 Fig. 4C demonstrates a 56% reduction in the 2.3-kilobase allele in tumor 1 compared with its corresponding normal mucosa. The 7.6-kilobase allele in tumor 7 lost approximately 50% when compared to the corresponding nontumorigenic tissue. There was no change in tumor 10, although this tumor carries a 64-base pair deleted coding sequence. This is also noted in an inverse relationship in the peak area between the 2.3- and 7.6-kilobase alleles; i.e., a reduction in the 2.3-kilobase intensity of the signal was accompanied by an amplification in the signal in the 7.6-kilobase band (tumor 1); with tumor 7, the opposite occurs. This type of DNA amplification (7.6-kilobase allele in tumor 1 or 2.3-kilobase allele in tumor 7) may be due to a rearrangement within a specific allele for the loss in the other affected allele of the nm23 gene.

**Fig. 1.** PCR-amplified nm23 coding sequences from tumor (T) and corresponding normal mucosa (N). PCR products were separated on 3.5% low melting agarose gel in TAE buffer and stained by ethidium bromide. 10 and 3 are the patient numbers. M, molecular weight marker in base pairs (left).

**Fig. 2.** Autoradiograph of sequence analysis showing the 64-base pair deletion (Δ) found in tumor 10.

**Fig. 3.** Sequence of PCR-amplified nm23 coding region. Italic underlined sequences, primers designed for PCR amplification. Highlighted underlined sequences, deleted 64 base pairs from tumor 10 and the in-frame stop codon TAG due to deletion. Italics under the wild-type sequences, deduced amino acid sequences of the mutant protein.
A total of 20 tumor specimens with their corresponding normal mucosa were analyzed without any knowledge of the pathology report. After completion of our analyses, we obtained the metastasis or nonmetastasis status of all the tumors. All the tumors were diagnosed as colorectal adenocarcinomas except specimen 23 which was an adenomatous polyp. The results are summarized in Table 1. Among 20 tumors examined, eight were pathologically diagnosed metastatic on the basis of the detection of adenocarcinomas in lymph nodes or other organs. Results presented in this study demonstrate that in 20 tumors, genetic alterations in the nm23-H1 gene detected either by reverse transcription-PCR sequencing or by allelic deletion occurred in four (1, 3, 7, and 10) of eight tumors. As shown in Table 1, all these tumors were found to have metastases in lymph nodes, lung, or liver. An unaltered nm23-H1 gene was found in four other tumors (6, 16, 17, and 20) associated with metastases. Sequence analyses of the coding sequence or Southern analyses of nm23-H1 revealed no changes in all tumors examined without any history of metastasis. A 64-base pair deletion was found in tumor 10, but no PCR product was detected in tumor 3. A possible explanation is that a rearrangement at either the 5' or the 3' end of the transcript of this tumor resulted in the inability to amplify the nm23 cDNA sequence, since PCR requires binding of both primers to the target template. In addition, we cannot rule out the possibility that mutations occurred at the promoter region of nm23, which probably would decrease the expression of this gene.

A significant allelic loss in the nm23 genome was observed in two tumors (1 and 7) associated with metastasis in comparison to the corresponding normal mucosa. Assuming that a 50% reduction in the intensity of the individual band (peak area is in the densometric data) is an indicator of LOH, the results shown in Fig. 4, A–C, probably indicate a LOH of nm23-H1 gene in tumors 1 and 7. These results are in accordance with the observations on the association of nm23-H1 allelic deletions with distant metastasis (15).

Even though we could detect mutations in only about 50% of metastatic tumors, it is important to note that no alteration was found in any of 12 nonmetastatic tumors. These results clearly indicate a correlation between the genetic alteration in nm23 and metastatic activity of tumors. It is important to note that our observations presented in this report also support the recent demonstration of a point mutation at amino acid 48 of nm23-H2 of a metastatic neuroblastoma converting leucine to valine (7).

There is a BglII recognition site in the nm23 mRNA sequence (3, 9). However, repeated Southern blot assays have shown only one allele (either 7.6- or 2.3-kilobase) in the genome, even in normal human samples (3). This RFLP indicates that the codon for amino acid 46 GAT may come from two different exons because our cDNA sequence clearly shows that the BglII site AGATCT exists in every nm23 mRNA. Unfortunately, the genomic organization of nm23-H1 has not yet been identified.

The diallelic RFLP of BglII digestion in the nm23-H1 gene has been reported (3, 9). From 68 unrelated individuals, the frequency of the 7.6-kilobase allele is 41% and that of the 2.3-kilobase allele is 59% (3). Through analysis of normal colon mucosa, white blood cells, and placenta, our results showed frequency of 34 and 66% for 7.6- and 2.3-kilobase alleles, respectively. However, in our six patients with single (mono) allele on the Southern blot analysis, only one showed the 2.3-kilobase band (17%). The other five represented 7.6-kilobase alleles, respectively. However, in our six patients with single (mono) allele on the Southern blot analysis, only one showed the 2.3-kilobase band (17%). The other five represented 7.6-kilobase alleles, respectively.

Table 1  Summary of genetic alterations in nm23-H1 gene in colorectal tumors

<table>
<thead>
<tr>
<th>Patient</th>
<th>RFLP (BglII)</th>
<th>Mutation</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Allelic loss</td>
<td>ND</td>
<td>Yes (lymph nodes)</td>
</tr>
<tr>
<td>7</td>
<td>Allelic loss</td>
<td>ND</td>
<td>Yes (lymph nodes)</td>
</tr>
<tr>
<td>10</td>
<td>Unaltered</td>
<td>64-base pair deletion</td>
<td>Yes (4 of 7 lymph nodes, liver, prostate)</td>
</tr>
<tr>
<td>3</td>
<td>Unaltered</td>
<td>No PCR product</td>
<td>Yes (19 of 20 lymph nodes, liver, lung)</td>
</tr>
<tr>
<td>6</td>
<td>Unaltered</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td>16</td>
<td>Unaltered</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>17</td>
<td>ND</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
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</tr>
<tr>
<td>5</td>
<td>Unaltered</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
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<td>ND</td>
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</tr>
<tr>
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<td>12</td>
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</tr>
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<td>14</td>
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</tr>
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<td>23</td>
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<td>No</td>
</tr>
<tr>
<td>24</td>
<td>Unaltered</td>
<td>ND</td>
<td>No</td>
</tr>
</tbody>
</table>

*None, no difference in the sequences between normal and tumor; ND, not determined.*
bands (87%), among which three were metastatic. It is unclear whether this characteristic inherited (or RFLP) linkage imbalance is associated with colorectal tumorogenesis, if it is, this imbalance may be used as a genetic marker for early diagnosis or genetic counseling for those people at high risk of colorectal cancer. A large number of colorectal tumor patients need to be surveyed to establish this correlation.

A 64-base pair deletion was observed in one of four tumors with metastasis examined. This deletion was at the COOH-terminal of the cDNA of nm23, as indicated in Fig. 3. Consequently, 21 amino acids were abolished in this tumor. Due to an early stop codon, a smaller nm23 gene product is expected to occur and consequently would impair the function of the gene.

Although the functional role of the nm23 gene has not been elucidated yet, its significantly high sequence homology with a NDPK (16) provides a new insight into the important involvement of nm23 in the regulatory processes of cellular growth and proliferation. Human erythrocyte NDPK consists of two polypeptide chains A and B. Chain A is identical with the human nm23-H1 gene product, while chain B has been shown to be identical to nm23-H2 (16, 17). A recent report indicates that a potential suppressor protein for differentiation of leukemic cells, designated as I-factor, is probably the nm23/NDPK protein (17). Thus, it strongly suggests a vital role of the nm23 gene in regulatory processes such as signal transduction, proliferation, and differentiation. Alterations in the nm23 gene in colorectal cancers (deletion in coding sequence or LOH as we present in this report) would modify these functions in a negative fashion. Mutation in the nm23 gene may increase the level of the gene product in tissues. Perhaps an enhanced nm23 expression in colon carcinomas (8) may be due to mutation(s) in this gene. Moreover, neuroblastoma and proliferating lymphoid cells have been reported to be associated with increased levels of the nm23 gene product and RNA expression (7, 18). Mutation(s) in nm23 may have a role in the causation of high levels of the nm23 protein. It will be of utmost importance to study the role of mutation(s) in nm23 in the functions of this gene, especially in metastasis.

Acknowledgments

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References


Second International Conference of Advances in the Biology and Clinical Management of Melanoma, February 22–25, 1995, Hyatt Regency Houston, Houston, TX. Contact: Carol Harrel, Conference Services, Box 131, 1515 Holcombe Blvd., Houston, TX 77030-4095. Telephone: (713) 792-2222.

Errata

An error has been found in the article by Wang et al., which appeared in the February 15, 1993 issue of Cancer Research (pp. 717–720). The primers in the “Materials and Methods” section (p. 717) are incorrect. They should be: hnm23c 5': GAGAATTCAATGTGGTCTGCCCTCC and hnm23c 3': GAGAATTCAATGTGGTCTGCCCCTCC.

There is an error in the article by Skrincosky et al., which appeared in the June 1, 1993 issue of Cancer Research (pp. 2667–2675). In the “Abstract” (p. 2667), the following section is incorrect: “125I-labeled polymerized galapitin. In a Western blot of A121 cell extracts separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, bound to a unique cellular protein having a molecular mass of 110 kDa.” It should read: “In a Western blot of A121 cell extracts separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 125I-labeled polymerized galapitin bound to a unique cellular protein having a molecular mass of 110 kDa.”

An error has been found in the article by Davies et al., in the May 1, 1993 issue of Cancer Research (pp. 2087–2091). The incorrect version of Fig. 4 was printed (p. 2089). Fig. 4 is reproduced correctly below.

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Fig. 4. Survival of mice bearing the Hu xenograft following treatment with 40 mg BB-94/kg/day or vehicle control as indicated. Mice were treated either from day 3 (A) or day 7 (B) after the introduction of xenograft until day 21 or death, whichever was first.
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