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Advances in Brief

Mutation in the *nm23* Gene Is Associated with Metastasis in Colorectal Cancer[1]

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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 as 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_r 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. Steggles, Northeastern Ohio Universities College of Medicine.

Reverse Transcription-PCR[4] 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 (Gibco/BRL, 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 hnm23 3′: GGGAATTCTGCGCCAGGGAGGATACAG, 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 *Eco*RI (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-^{35}S]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 *Bgl*II and separated on 0.8% agarose gel and then transferred to Nitran membrane (13). The blots were hybridized with an α-^{32}P-labeled 533-base pair-*nm23-H1* probe (entire coding sequence of normal colon mucosa prepared 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

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[4] The abbreviations used are: PCR, polymerase chain reaction; cDNA, complementary DNA; LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; NDPK, nucleoside diphosphate kinase.

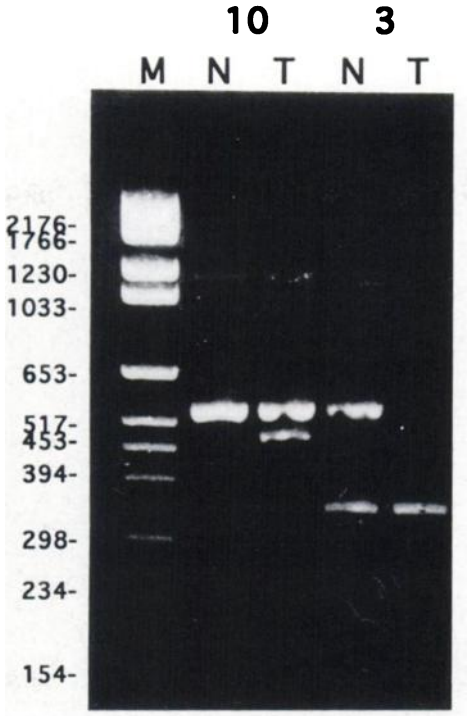

Fig. 1. PCR-amplified *nm23* coding sequences from tumor (*T*) and corresponding normal mucosa (*N*). PCR products were separated on 3.5% low melting agarose gel in TAE buffer and stained by ethidium bromide. 10 and 3 are the patient numbers. *M*, molecular weight marker in base pairs (*left*).

band. The smaller band was not observed in the corresponding normal mucosa. Only the smaller 350-base pair band was amplified in tumor 3 without the expected 533-base pair product. As shown in Fig. 1, the expected 533-base pair product was present in the corresponding normal mucosa, but it is interesting to note that along with the normal product, the smaller product is also present in the normal mucosa.

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 *Bgl*II 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. 4*A*). 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. 4*B*). 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 *Bgl*II RFLP (data not presented).

Comparing the densitometric analysis by a Bio-Rad video densitometer model 620 Fig. 4*C* 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. 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.

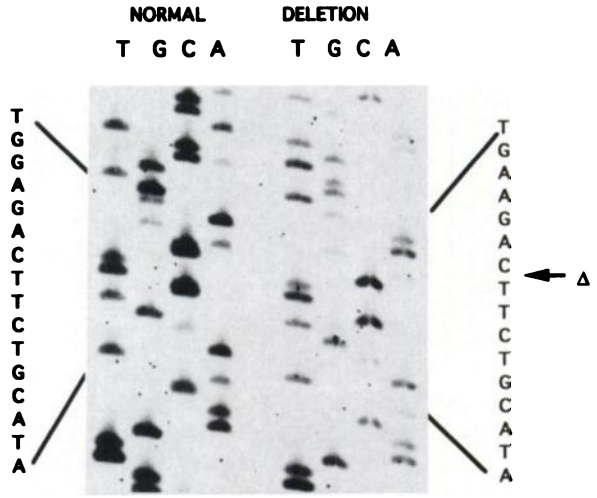

Fig. 2. Autoradiograph of sequence analysis showing the 64-base pair deletion (←Δ) found in tumor 10.

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                TGC TGC GAA CCA CGT GGG TCC CGG GCG CGT TTC GGG TGC

TGG CGG CTG CAG CCG GAG TTC AAA CCT AAG CAG CTG GAA GGA ACC

ATG GCC AAC TGT GAG CGT ACC TTC ATT GCG ATC AAA CCA GAT GGG
Met Ala Asn Cys Glu Arg Thr Phe Ile Ala Ile Lys Pro Asp Gly

GTC CAG CGG GGT CTT GTT GGA GAG ATT ATC AAG CGT TTT GAG CAG
Val Gln Arg Gly Leu Val Gly Glu Ile Ile Lys Arg Phe Glu Gln

AAA GGA TTC CGC CTT GTG GGT CTG AAA TTC ATG CAA GCT TCC GAA
Lys Gly Phe Arg Leu Val Gly Leu Lys Phe Met Gln Ala Ser Glu

GAT CTT CTC AAG GAA CAC TAC GTT GAC CTG AAG GAC CGT CCA TTC
Asp Leu Leu Lys Glu His Tyr Val Asp Leu Lys Asp Arg Pro Phe

TTT GCC GGC CTG GTG AAA TAC ATG CAC TCA GGG CCG GTA GTT GCC
Phe Ala Gly Leu Val Lys Tyr Met His Ser Gly Pro Val Val Ala

ATG GTC TGG GAG GGG CTG AAT GTG GTG AAG ACG GGC CGA GTC AGG
Met Val Trp Glu Gly Leu Asn Val Val Lys Thr Gly Arg Val Met
                                        Thr

CTC GGG GAG ACC AAC CCT GCA GAC TCC AAG CCT GGG ACC ATC CGT
Leu Gly Glu Phr Asn Pro Ala Asp Ser Lys Pro Gly Thr Ile Arg

GGA GAC TTC TGC ATA CAA GTT GGC AGG AAC ATT ATA CAT GGC AGT
Gly Asp Phe Cys Ile Gln Val Gly Arg Asn Ile Ile His Gly Ser
   Ser Ala Tyr Lys Leu Ala Gly Thr Leu Tyr Met Ala Val

GAT TCT GTG GAG AGT GCA GAG AAG GAG ATC GGC TTG TGG TTT CAC
Asp Ser Val Glu Ser Ala Glu Lys Glu Ile Gly Leu Trp Phe His
 Ile Leu Trp Arg Val Gln Arg Arg Arg Ser Ala Cys Gly Phe Thr

CCT GAG GAA CTG GTA GAT TAC ACG AGC TGT GCT CAG AAC TGG ATC
Pro Glu Glu Leu Val Asp Tyr Thr Ser Cys Ala Gln Asn Trp Ile
 Leu Arg Asn Trp stop

TAT GAA TGA CAG GAG GGC AGA CCA CAT TGC TTT TCA CAT CCA
Tyr Glu Stop
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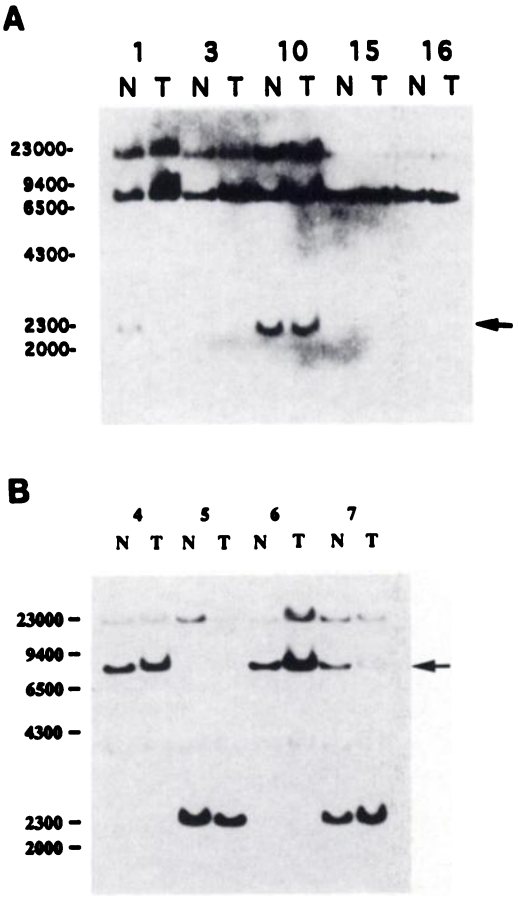
Fig. 3. Sequence of PCR-amplified *nm23* coding region. *Italic underlined sequences*, primers designed for PCR amplification. *Highlighted underlined sequences*, deleted 64 base pairs from tumor 10 and the in-frame stop codon TAG due to deletion. *Italics* under the wild-type sequences, deduced amino acid sequences of the mutant protein.



C

Allele kb	Area in Peak 1 N	1 T	10 N	10 T	7 N	7 T
2.3	3.4	1.5	3.1	3.9	5.8	8.2
7.6	2.9	6.7	3.2	3.2	0.8	0.4

Fig. 4. Southern blot analysis of DNA from tumors (*T*) and their corresponding normal mucosa (*N*). ^{32}P-labeled 533-base pair entire coding region of *nm23* cDNA was used to hybridize *Bgl*II-digested genomic DNA. Numbers of specimens are given on each pair of DNA. *Left*, molecular weight as base pairs. *Arrow*, position of allelic deletion. *A* and *B* are two independent experiments. *C*, densitometric (Bio-Rad) analysis of *Bgl*II-*nm23* restriction pattern in tumor (*T*) and normal mucosa (*N*) DNA. *1*, *10*, and *7*, specimen numbers.

Discussion

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 adenometous 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 *Bgl*II 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 sequencing clearly shows that the *Bgl*II site AGATCT exists in every *nm23* mRNA. Unfortunately, the genomic organization of *nm23-H1* has not yet been identified.

The diallelic RFLP of *Bgl*II 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

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

Patient	RFLP (*Bgl*II)	Mutation	Metastasis
1	Allelic loss	None[a]	Yes (lymph nodes)
7	Allelic loss	ND	Yes (lymph nodes)
10	Unaltered	64-base pair deletion	Yes (4 of 7 lymph nodes, liver, prostate)
3	Unaltered	No PCR product	Yes (19 of 20 lymph nodes, liver, lung)
6	Unaltered	ND	Yes
16	Unaltered	None	Yes
17	ND	None	Yes
20	ND	None	Yes
2	Unaltered	ND	No
4	Unaltered	ND	No
5	Unaltered	ND	No
8	Unaltered	ND	No
11	Unaltered	None	No
12	Unaltered	None	No
14	ND	None	No
15	Unaltered	None	No
18	ND	None	No
22	Unaltered	None	No
23	ND	None	No
24	Unaltered	ND	No

[a] 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 tumorigenesis, if it is, this imbalance may be used as a genetic marker for early diagnosis or genetic consulting 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 (1, 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

The authors are indebted to our Cleveland Clinic colleagues, Drs. James M. Church and Jeffrey Milsom, Department of Colorectal Surgery, and Claudio Fiocchi, Department of Immunology, for providing us with the specimens and to Dr. Ronald M. Bukowski, Experimental Therapeutics, for his support and encouragement. We thank Dr. A. W. Steggles, Northeastern Ohio Universities College of Medicine, for providing us the human placenta.

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Sixth International Symposium on Luminescence Spectrometry in Biomedical Analysis—Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, June 5–7, 1994, Congress Centre Oud Sint-Jin, Belgium. Contact: Dr. Willy R. G. Baeyens, Symposium Chairman, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Lab. of Drug Quality Control, Harelbekestraat 72, B-9000 Ghent, Belgium. Telephone: 32-9-221-89-51; FAX: 32-9-221-41-75.

Sixteenth International Cancer Congress (UICC), October 30–November 5, 1994, New Delhi, India. Contact: Congress Secretariat, Sixteenth International Cancer Congress, Tata Memorial Centre, Parel, Bombay 400 012, India. Telephone: 91-22-412-9399 (Direct) and 91-22-414-6685 Ext. 4265/4270; FAX: 91-22-413-9318 and 91-22-414-6937.

Second International Conference of Advances in the Biology and Clinical Management of Melanoma, February 22–25, 1995, Hyatt Regency Houston, Houston, TX. Contact: Carol Harreld, 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′: AAGAATTCGGGTGCTGGCGGCTG and hnm23c 3′: GAGAATTCAATGTGGTCTGCCCTCC.

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: "^{125}I-labeled polymerized galaptin. 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, ^{125}I-labeled polymerized galaptin 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.

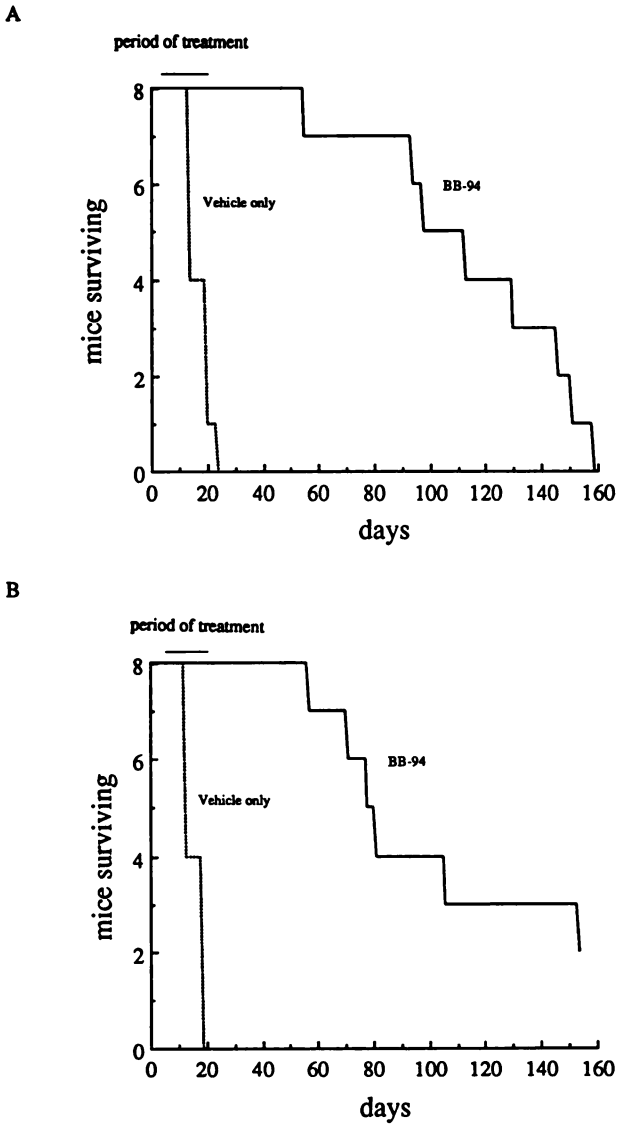

Fig. 4. Survival of mice bearing the Hu xenograft following treatment with 40 mg BB-94/kg/day or vehicle control as indicated. Mice were treated either from day 3 (*A*) or day 7 (*B*) after the introduction of xenograft until day 21 or death, whichever was first.

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