Overexpression of *nm23*-H1 and *nm23*-H2 genes in colorectal carcinomas and loss of *nm23*-H1 expression in advanced tumour stages

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Abstract

Although a reduced expression of *nm23* has been shown to correlate with a high metastatic potential in some human cancers, in colorectal cancers, conflicting data have been reported. As there are two homologous genes, *nm23*-H1 and *nm23*-H2, which encode the A and B subunits of nucleoside diphosphate kinase, efficient and simplified techniques were designed to selectively study *nm23*-H1 and -H2 expression in 35 colorectal cancers at both the protein and mRNA levels by immunoblotting, immunohistochemistry, and reverse transcription polymerase chain reaction (RT-PCR) using specific antibodies and primers. Nm23-H1 and Nm23-H2 proteins were overexpressed in tumours compared with adjacent mucosa. This overexpression was lost, however, in some advanced cases: 89% and 81% of TNM (tumour, node, metastases) stages 0-II showed Nm23-H1 and -H2 overexpression, respectively, which significantly differed from 47% and 38% of stage III-IV tumours. Similar results were seen with *nm23*-H1 mRNA. Heterogenous labelling of tumoral cells was seen by immunohistological staining. This suggests a dichotomy: an overexpression of *nm23*-H1 and -H2 linked to early stages of cancer and a loss of *nm23*-H1 overexpression seen in more advanced stages. Therefore specific *nm23*-H1 determination should be evaluated as a prognostic factor in human colorectal carcinoma. (Gut 1995; 37: 712-720)

Keywords: Nm23-H1, Nm23-H2, NDP kinase, metastasis, colorectal cancer.

Improving the outlook for colorectal cancer requires better diagnostic and prognostic facilities, screening programmes in high risk subjects, and more effective treatment for advanced disease.¹ As pathological criteria are not sufficient, biochemical and molecular biology techniques may be useful in this staging process. Recently, a range of abnormalities has been identified in colorectal cancer including *ras* mutations and deletions of gene sequences on chromosomes 5, 17, and 18.² Allelic losses on chromosomes 17 and 18 are associated with a poor prognosis independent of conventional pathological staging³ as does a combination of *p53* overexpression and *c-k1-ras* gene mutation.⁴ In addition, *nm23* gene

changes or expression values were recently discussed as prognostic factors in colorectal cancers (see below).

The *nm23* gene, originally identified on the basis of its reduced expression in a highly metastatic murine melanoma cell subline (K-1735), was proposed as a metastasis suppressor gene.⁵ In addition, a second gene, *nm23*-H2, has been identified,⁶ 83% identical to *nm23* now referred to as *nm23*-H1. *nm23*-H1 and *nm23*-H2 genes are located on chromosome 17 q22, 4 kb apart,^{7 8} and code, respectively, for the A and B subunits of the nucleoside diphosphate (NDP) kinases.⁹ These enzymes are hexamers formed of various proportions of A and B monomers⁹ and are responsible for the conversion of ribo and deoxyribonucleoside diphosphates to the corresponding triphosphates. The metastatic potential suppressor function of the *nm23*-H1 gene has been proposed based on experiments, in which transfection of *nm23*-H1 cDNA into highly metastatic cell lines derived from a murine melanoma and a human breast carcinoma, reduced their metastatic potential.^{10 11} In addition, reduced *nm23* expression was associated with aggressive clinical courses in several, but not all, human tumour cohorts, including human breast carcinomas,¹²⁻¹⁴ primary hepatocellular carcinomas,^{15 16} melanoma metastases,¹⁷ and ovary carcinomas.¹⁸ Another aspect relating *nm23* to cancer is the report of its overexpression in several types of solid tumours compared with the matched normal tissue.¹⁹⁻²¹

In colorectal cancer, conflicting data have been reported regarding *nm23* expression and tumour aggressiveness. Somatic allelic deletion of the *nm23* gene has been reported in advanced cases of colon cancer,^{7 22 23} although not found by others.^{24 25} In addition, allelic and intragenic deletion of *nm23*-H1 have been found in four of eight tumours with metastases, while no change was seen in non-metastatic tumours.²⁶ Modified expression of *nm23* was also described. Yamaguchi *et al*²⁷ reported reduced expression of *nm23*-H1 mRNA in patients with colorectal cancer associated with distant and hepatic metastases while Haut *et al*²¹ and Myeroff *et al*,²⁴ failed to find an association between *nm23* expression and metastatic spread in this cancer. In addition, Royds *et al*²⁸ suggested that Nm23 could have a protective effect regarding the relative risk of dying from colorectal cancer.

A reason for these discrepancies could be that most studies do not discriminate between

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the expression of nm23-H1 and nm23-H2 for which antibodies and full length cDNA probes cross react. This prompted us to analyse the selective expression of nm23-H1 and nm23-H2 in colorectal cancer, using discriminating tools, at both the mRNA level, by reverse transcription polymerase chain reaction (RT PCR) using a semi-quantitative coamplification procedure, and the protein concentration, by immunoblotting, using specific antibodies. Moreover, immunohistochemical analysis was performed in some cases with the aim of studying the intracellular and intratissular localisation of both Nm23 proteins.

Methods

Patients and tissue samples

Specimens of 35 colorectal carcinomas and the adjacent normal mucosa were obtained, before any fixative processing, from patients operated on in the Department of Digestive Surgery, Hôpital Saint-Antoine, Paris. Specimens of five hepatic metastases of colonic origin and three acute colitis were also analysed. Each fresh sample was immediately frozen in liquid nitrogen, divided into sections for immunoblotting analysis and RNA extraction, and stored at -80°C . After fixation, colorectal tissue specimens and liver metastases were sampled according to currently used protocols to classify the neoplastic lesions and define tumour characteristics and some specimens were taken for immunohistochemical analysis. The pathological and clinical staging was performed according to the TNM (tumour, node, metastases) classification of the International Union Against Cancer.²⁹

Immunoblotting

Frozen tissues of each specimen were homogenised (Ultra-turrax T25 homogeniser, full power 2×30 sec) in a 10 mM TRIS-HCl, pH 7.5 buffer containing 10% glycerol, 1 mM EDTA, 1 mM DTT, and protease inhibitors (1 mM PMSF, 1 $\mu\text{g/ml}$ of antipain and pepstatin, and 15 $\mu\text{g/ml}$ of benzamidine). The protein concentration of the samples was determined according to Bradford.³⁰ Lysates (5 μg protein) were electrophoretically separated on SDS resolving gels containing 10% polyacrylamide according to Laemmli,³¹ transferred in 25 mM potassium phosphate buffer, pH 7, to an Immobilon membrane (Millipore), and subsequently fixed to the membrane by incubation at room temperature for 45 minutes in the same buffer containing 0.2% glutaraldehyde according to Van Eldick.³²

Nm23-H1 and H2 proteins (NDP kinases A and B) were detected using, in the primary reaction, polyclonal antibodies raised against NDP kinase A and B purified from human erythrocytes as described⁹ and an anti-NDP kinase A monoclonal antibody (HA-37.6) raised by Hybridolab, Pasteur Institute, Paris, using the same antigen. A peroxidase conjugated antirabbit (polyclonal) IgG antibody or a peroxidase conjugated antimouse

(monoclonal) IgG antibody was used in the secondary reaction.

The polyclonal antiserum directed against NDP kinase A was affinity purified by chromatography through a column (Hi Trap from Pharmacia) to which the recombinant NDP kinase A denatured in guanidinium chloride was bound (IL, MLL, unpublished data). It recognises preferentially the A polypeptide and to a lesser extent the B polypeptide. Electrophoretic migration of the A and B monomers gives clearly distinct bands at positions corresponding to 20.5 and 19 kilodaltons (kDa), respectively.⁹ Purified recombinant A and B human enzymes (MLL, IL, unpublished results) were used as standard. Their positions have been determined by reference to the low range molecular mass markers purchased from BioRad (not shown).

The polyclonal anti-NDP kinase B antiserum was depleted of antibodies reacting with NDP kinase A epitopes by filtration through a column coupled to the recombinant A enzyme. It is specific for the H2 protein (B peptide) without cross reactivity with the Nm23-H1 protein. The monoclonal antiserum directed against the A enzyme is specific for the Nm23-H1 protein. Immunodetection was performed using the Enhanced Chemiluminescence Detection System of Amersham (Les Ulis, France).

Immunohistochemistry

Immunohistochemical detection of Nm23 proteins was performed on 5 μm paraffin wax embedded sections previously fixed either in 4% formalin, or aqueous Bouin solution obtained from 14 cases of colorectal adenocarcinomas with the respectively matched 'normal' mucosa and from one case of acute colitis. All steps were carried out at room temperature. Sections were dewaxed, rehydrated, and treated for 20 minutes with 5% creamless milk to prevent non-specific binding. They were then incubated for 30 minutes either with the polyclonal anti-NDP kinase A or anti-NDP kinase B antibodies. Immunohistochemistry followed a three step procedure, using peroxidase and aminoethyl carbazole. Sections were counter stained with haemalin for two minutes.

RT PCR amplification

Total RNA of each specimen was prepared by extraction with guanidine isothiocyanate according to Chirgwin *et al*³³ and determined to be intact by migration on 1% agarose gels and staining with ethidium bromide. Two μg of total RNA from different specimens were reverse transcribed using random hexanucleotides as primers as described elsewhere.³⁴ After determining that the appropriate number of PCR cycles for detection and quantification of nm23-H1, nm23-H2, and β -actin DNA fragments was between 25 and 35 cycles, first strand cDNA was used directly for 30 cycles of PCR amplification using Taq polymerase (Beckman, USA) and 0.4 μCi

TABLE I Clinical features and tumour characteristics in patients with colorectal cancer

Patient	Age (y)	Sex	Primary tumour localisation	Adenocarcinoma – histological type	Metastasis	Astler-Coller classification	TNM stage
1	74	Male	Left colon	Intraepithelial	–	A	0
2	53	Male	Left colon	Well differentiated	–	A	I
3	46	Female	Rectum	Well differentiated mucinous	–	B1	I
4	79	Female	Rectum	Well differentiated	–	B1	I
5	63	Male	Left colon	Well differentiated	–	B1	I
6	50	Female	Left colon	Well differentiated	–	B1	I
7	60	Female	Left colon	Well differentiated	–	B2	II
8	83	Male	Rectum	Well differentiated	–	B2	II
9	43	Male	Rectum	Well differentiated	–	B2	II
10	83	Female	Left colon	Well differentiated	–	B2	II
11	79	Female	Rectum	Well differentiated	–	B2	II
12	64	Male	Rectum	Well differentiated	–	B2	II
13	86	Female	Right colon	Well differentiated	–	B2	II
14	51	Male	Left colon	Well differentiated mucinous	–	B2	II
15	56	Male	Left colon	Well differentiated	–	B2	II
16	71	Male	Rectum	Well differentiated	–	B2	II
17	73	Female	Rectum	Well differentiated mucinous	–	B2	II
18	83	Male	Left colon	Well differentiated	–	B2	II
19	61	Female	Rectum	Moderately differentiated	LN	C1	III
20	77	Male	Rectum	Well differentiated	LN	C2	III
21	87	Female	Right colon	Well differentiated	LN, P	C2	III
22	68	Male	Right colon	Moderately differentiated	LN	C2	III
23	67	Female	Rectum	Well differentiated	LN	C2	III
24	82	Female	Rectum	Well differentiated mucinous	LN	C2	III
25	59	Male	Rectum	Well differentiated	LN	C2	III
26	78	Female	Rectum	Well differentiated	LN	C2	III
27	51	Male	Rectum	Well differentiated	LN	C2	III
28	73	Female	Rectum	Well differentiated	H	D	IV
29	71	Male	Right colon	Well differentiated	LN, H	D	IV
30	67	Female	Left colon	Well differentiated	LN, H	D	IV
31	64	Male	Rectum	Well differentiated mucinous	LN, H	D	IV
32	81	Female	Right colon	Moderately differentiated	LN, H	D	IV
33	52	Female	Left colon	Well differentiated mucinous	LN, H	D	IV
34	86	Male	Rectum	Well differentiated	H	D	IV
35	57	Male	Left colon	Well differentiated	H	D	IV

LN: lymph node metastasis; H: hepatic metastasis; P: peritoneal metastasis.

[α - 32 P]dCTP per assay. Incorporation of radioactive dCTP was equivalent in *nm23*-H1 and H2 as their C+G contents are similar. Three pairs of *nm23*-H1, *nm23*-H2 and β -actin (internal standard) primers were used together for amplification. The primers were designed to amplify the entire *nm23*-H1 and *nm23*-H2 coding sequence. There were: *nm23*-H1 sense: 5' CCATGGCCAACTGTGAGCGT 3' and antisense: 5' GCCTCCTGTCAATTCATAGAT 3'; *nm23*-H2 sense: 5' CCATGGC-CAACCTGGAG CGC 3' and antisense: 5' GTCCACCTCTTATTCATAGAC 3'. The β -actin primers were sense: 5' ATCAT-GTTTGAGACCTTCAA 3' and antisense: 5' TTGCGCTCAGGAGGAGCAAT 3'. After 5 minutes of denaturation at 94°C, 30 PCR cycles including denaturation for 30 seconds at 94°C, annealing for one minute at 60°C, and polymerisation for two minutes at 72°C were performed. At the end of the 30 cycles, a supplementary step of polymerisation at 72°C for five minutes was added. The PCR products were subsequently incubated at 37°C with *Pst* I restriction endonuclease (Biolabs, France), which digests specifically *nm23*-H1 amplified cDNA and permits discrimination between *nm23*-H1 and *nm23*-H2 PCR fragments. Only the *nm23*-H1 sequence has the specific digestion site for *Pst* I (CTGCA^vG) giving two fragments of 302 and 186 bp while *nm23*-H2 is 488 bp long. The PCR products were electrophoretically separated on 2% agarose gels and transferred to a nylon membrane (Hybond-N+, Amersham, Les Ulis, France). The radioactivity incorporated to in each PCR product was determined using a Phosphor Imager (Molecular Dynamics, USA) or a Packard Instant Imager. We verified that the PCR products corresponded to *nm23*-H1 and

-H2 by hybridisation with specific 22-mer oligonucleotide probes corresponding to the most divergent part of both sequences (*nm23*-H1 probe: 5' AGCTTGCATGAA-TTTCAGACCA 3' and *nm23*-H2 probe: 5' GGCCCGGAGGAAGTTCATGGCC 3') labelled with T4 polynucleotide kinase.

Statistical analysis

For statistical analysis the χ^2 test or the Student's *t* test for paired and unpaired values were performed. Differences were taken as significant when *p* was less than 0.05.

Results

PATIENTS AND TUMOUR CHARACTERISTICS

The colorectal primary tumour tissue and corresponding normal mucosa were obtained from colectomy or coloproctectomy specimens of 18 male and 17 female patients with a mean age of 68 years (range, 43–87). Table I summarises the clinical features and tumour characteristics of the specimens analysed.

EXPRESSION OF Nm23-H1 AND Nm23-H2 PROTEINS

Immunoblotting

The expression of Nm23-H1 and Nm23-H2 proteins was analysed by western immunoblotting using specific antibodies (Fig 1). Two bands migrating at positions corresponding to 20.5 and 19 kDa are the Nm23-H1 and Nm23-H2 proteins, respectively. A higher expression of both proteins is seen in tumour tissues compared with normal mucosa, in most

Figure 1: Immunoblotting of tissue extracts (5 µg of protein) from: an acute segmentary colitis case (C), four representative colorectal cancers (T), a metachronic liver metastasis from colonic origin (LM), and the respective matched normal mucosa (N) or the adjacent normal liver tissue (NL). The numbers correspond to the cases listed in Table I. Detection of Nm23-H1 and H2 proteins was performed using an affinity purified anti-NDP kinase A polyclonal antiserum (A), an anti-anti-NDP kinase A monoclonal antibody (B), and an anti-NDP kinase B polyclonal antiserum (C). One and 10 ng of purified recombinant A and B human enzymes (A rec and B rec) were used as standard. M+ and M- indicate cases with and without metastases, respectively.

cases. Table II summarises the results. Twenty four cases (69%) showed Nm23-H1 expression that exceeded that of the matched normal mucosa. Nineteen of 32 cases analysed (59%), showed Nm23-H2 overexpression. The tumours that did not show Nm23-H1 or H2 overexpression had an expression level that was similar in most cases to the adjacent mucosa. Three cases of acute colitis were also analysed and all three showed a similar or lower level of Nm23-H1 and Nm23-H2 expression compared with the normal tissue (not shown).

Eighty nine per cent (16 of 18) of colorectal cancer patients TNM stages 0–II (N0, M0) showed overexpression of the Nm23-H1 protein. In contrast, only 47% (eight of 17) of stage III–IV (N1 or M1) cases showed Nm23-H1 overexpression with a highly significant difference between the two groups ($p < 0.01$). Eighty one per cent of stage 0–II cases also showed Nm23-H2 overexpression compared with 38% of stage III–IV cases ($p < 0.05$). Thus, 82% (nine of 11) and 77% (10 of 13) of cases that did not show respectively Nm23-H1 and Nm23-H2 overexpression, had advanced stage cancers. Most cases that showed Nm23-H1 overexpression also showed Nm23-H2 overexpression although less noticeable than that for Nm23-H1.

Four of the five cases of colorectal liver metastasis (one synchronic, four metachronic) showed a lower level of Nm23-H1 expression

compared with the adjacent hepatic tissue (three of four) or to the primary colonic cancer (one of one). This did not result from a higher level of expression of Nm23-H1 protein in the liver compared with colonic normal mucosa, as expression of Nm23-H1 is similar in both tissues (data not shown). Two of three colorectal metachronic liver metastases analysed, showed a lower level of Nm23-H2 expression compared with the adjacent hepatic tissue, although the differences were less noticeable than that for Nm23-H1 (Fig 1).

Immunohistochemistry

Immunohistological localisation of Nm23-H1 and H2 proteins was studied in one case TNM stage 0 (case 1), four cases stage I (case 2, 3, 4, and 5), two cases stage II (cases 7 and 10), four cases stage III (cases 20, 22, 23, and 24), and three cases stage IV (28, 29, and 30) of colorectal adenocarcinomas.

Adjacent colorectal mucosa – in normal mucosa, non-mucinous epithelial cells of the surface epithelium and of the acini localised to the superficial third of the mucosa were weakly positive in the cytoplasm for both proteins (Fig 2(A)). No positivity was detected in the mucinous secretory cells. The reaction in non-mucinous epithelial cells has a delicate

TABLE II Expression of Nm23-H1 and Nm23-H2 proteins in 35 patients with colorectal cancer

Patient	TNM stage	Nm23-H1		Nm23-H2
		(p)	(m)	(p)
1	0	+	+	+
2	I	+	+	+
3	I	++	+	+
4	I	++	++	+
5	I	+	+	+
6	I	++	+	+
7	II	+	++	+
8	II	+	+	+
9	II	–	–	+
10	II	++	++	+
11	II	++	+	+
12	II	+	+	+
13	II	++	++	+
14	II	++	++	++
15	II	+	+	+
16	II	++	++	++
17	II	–	–	–
18	II	–	–	–
19	III	–	–	–
20	III	–	–	–
21	III	+	+	++
22	III	+	++	–
23	III	–	–	–
24	III	–	–	–
25	III	++	+	+
26	III	–	–	–
27	III	+	++	+
28	IV	+	++	+
29	IV	–	–	–
30	IV	–	–	–
31	IV	++	+	+
32	IV	+	+	+
33	IV	–	–	–
34	IV	++	++	+
35	IV	–	–	–
		8/17 (47%)*		6/16 (38%) ‡

(p): anti-Nm23-H1 or -H2 polyclonal antibody; (m): anti-Nm23-H1 monoclonal antibody.

–: signal less intense than in matched normal tissue;

–: signal intensity similar to that in matched normal tissue.

+: signal more intense than in matched normal tissue;

++: very strong signal more intense than in matched normal tissue.

‡: The number of cases overexpressing Nm23-H1 or Nm23-H2 proteins compared with the total number of cases in the groups of TNM 0–II and TNM III–IV ‡ stages are indicated. Statistical evaluation of the difference between the two groups of the TNM stages: * $p < 0.01$; ** $p < 0.05$.

Figure 2: Immunohistochemical localisation of Nm23 proteins H1 (A, B, C) and H2 (D) in normal mucosa (A) and adenocarcinoma (B, C, D) of the colon. Original magnification: $\times 200$. (A) normal colonic mucosa showing intracytoplasmic localisation of Nm23-H1 in non-mucinous epithelial cells of the surface epithelium and superficial acini (upper half). (B, C, D) Heterogeneity of the overexpression of Nm23 proteins in the same case of colonic adenocarcinoma. Mild (B) and high (C) expression of Nm23-H1; higher expression of Nm23-H2.

granular pattern and is found in the apex of the cells. Background staining was higher with the anti-NDP kinase B/Nm23-H2 than with the anti-NDP kinase A/Nm23-H1 antibody. No difference of labelling was seen in the inflammatory mucosa chosen as a control.

Neoplastic tissues – immunoreactivity for both proteins was localised to the cytoplasm of non-mucinous secretory tumoral cells (Fig 2(B), (C), (D)). Nuclear positivity was seen only in a few cases, with both anti-NDP kinase A and anti-NDP kinase B polyclonal antibodies and therefore was not considered as a significant signal. The level of Nm23 proteins expression in neoplastic cells was compared in each case with their expression in the adjacent non-neoplastic colorectal mucosa.

Immunohistochemical analysis of tumour tissues was considered as non-significant in two cases (cases 22 and 29) that presented large areas of necrosis. In the other cases, the intensity of the coloured reaction with the anti-NDP kinase A antibody and the number of carcinomatous crypts that gave immunoreactivity, varied greatly from one case to the

other, as well as from one area to the other in the same tumour (Fig 2(B), (C)). This heterogeneity of labelling was also seen with the anti-NDP kinase B antibody. Higher labelling signals of Nm23-H1 protein compared with the matched normal mucosa were seen in 10 of 12 cases (83%), although the percentage of the labelled carcinomatous acini was variable. The two remaining cases were superficial tumours; one of them (case 3) was a mucinous adenocarcinoma and the other (case 1), an intra-epithelial carcinoma developed in an adenoma. We also tested a benign adenoma associated with an adenocarcinoma (from case 29) and saw no overexpression of Nm23-H1 in the adenomatous crypts compared with the non-neoplastic colorectal mucosa. The expression of Nm23-H2 protein was studied in eight of 14 cases. Two cases were negative and corresponded to the negative cases for Nm23-H1 expression. In four of six positive cases (cases 4, 20, 24, and 28), the expression of Nm23-H2 was more intense than the expression of Nm23-H1 in the tumour tissue: the intensity of the reaction was higher, as was the number of labelled carcinomatous acini (Fig 2(D)). No correlation was seen between the intensity of the reaction in the tumorous areas and the intensity of the labelling of the colorectal mucosa in each case. Because of the heterogeneity of the labelling (10 to 100% of the carcinomatous acini being labelled), it was not possible to find a correlation between immunohistochemical signals and TNM cancer staging.

In summary, in non-mucinous epithelial tumour tissues, a higher staining and a heterogeneity of labelling were seen for both Nm23-H1 and Nm23-H2 compared with adjacent colonic mucosa.

EXPRESSION OF *nm23-H1* AND *nm23-H2* mRNA DETECTED BY RT PCR

The RT PCR technique was selected because it allows for the detection in the same specimen of *nm23-H1* and *nm23-H2* mRNAs compared with an internal standard. Figure 3 shows experiments performed to set up the technical procedure. We verified that the chosen couple of primers permitted the specific amplification of *nm23-H1* and *nm23-H2* fragment with no cross amplification, by hybridisation with specific *nm23-H1* and H2 probes (Fig 3(A)). The quantification of radioactive signals showed that the separate amplification of *nm23-H1*, *nm23-H2*, and β -actin gave signals equivalent to those obtained by simultaneous amplification (Fig 3(B)). The cases that did not present a reasonably constant level of β -actin cDNA amplification between tumour and normal tissues were eliminated, thus expression of the target genes together with that of the β -actin gene have been analysed in 28 cases. No amplification of genomic DNA was seen under these conditions (data not shown). Figure 4 shows some representative examples of the expressions of the *nm23-H1*, *nm23-H2*, and β -actin genes in tumour and normal matched tissues.

Figure 3: Detection of nm23-H1 and H2 mRNA by RT-PCR. (A) nm23-H1 and nm23-H2 PCR fragments were spotted onto Hybond-N+ membrane (Amersham) and revealed using the specific probes labelled with T4 polynucleotide kinase. (B) Images obtained with the Packard System analyser from the 30 cycles PCR products of the same tissue specimen from a representative case of colon cancer (case 7). (1) nm23-H2 PCR fragment, (2) nm23-H1 PCR fragment, (3) nm23-H1 PCR product digested with Pst I restriction endonuclease giving two fragments of 302 and 186 bp, (4) nm23-H1 and H2 amplified simultaneously and incubated with Pst I, (5) β-actin PCR fragments in tumour (T) and normal matched mucosa (N), (6) PCR fragments obtained after simultaneous amplification of the target genes and β-actin followed by incubation with Pst I in tumour (T) and normal matched mucosa (N).

Table III summarises the results of quantification. The mean (SEM) of the nm23-H1/β-actin ratio in TNM stage 0–II tumours was 0.46 (0.11), significantly higher than the respective values for the corresponding matched mucosa: 0.29 (0.08) ($p < 0.01$) while the mean nm23-H1/β-actin ratio of stage III–IV tumours was not significantly different to the ratio corresponding to the adjacent non-neoplastic mucosa. No significant differences were seen for nm23-H2. It should be emphasised that the relative expression of nm23-H2 was almost always higher than that of nm23-H1 in both tumour and normal specimens.

The threshold chosen to define overexpression of the target genes is a tumour/normal

tissue (Tu/Nt) relative expression ratio ≥ 1.2 . Considering the Tu/Nt ratio as a function of TNM grading, 71% (10 of 14) of colorectal cancer patients TNM stages 0–II (N0, M0) showed overexpression of the nm23-H1 mRNA while this was the case of only 43% (six of 14) of stage III–IV (N1 or M1) cases ($p < 0.05$). The nm23-H1 Tu/Nt ratio, mean (SEM) for stage 0–II cases was 2.34 (0.54) ($n = 14$) significantly different from 1.00 (0.15) ($n = 14$) for stage III–IV cancers ($p < 0.05$). Therefore, as seen at the protein level, most cases (67%; eight of 12) that did not show nm23-H1 mRNA overexpression had more advanced cancers. Even though there was a similar tendency for nm23-H2 the Tu/Nt ratio in stage 0–II and in stage III–IV cases are not significantly different. Two cases of acute colitis showed an absence of overexpression of nm23-H1 and nm23-H2 mRNA compared with normal tissues (data not shown). The data shown in Table III, on the relative expressions of the target genes in tumours to those in normal tissues are depicted in Fig 5 as a function of pathological TNM staging. The lack of overexpression seen in advanced (TNM III–IV stages) was even more pronounced when we consider only TNM IV stage (metastasis to distant organs; Nm23-H1 Tu/Nt ratio, mean (SEM) 0.73 (0.23)).

Discussion

In this study, the selective expression levels of both the protein and mRNA of nm23-H1 and nm23-H2 isoforms were analysed in colorectal cancers using discriminating probes and antibodies. Most tumours without known metastasis exhibited an increased expression of the two Nm23 isoforms in tumour cells compared with normal mucosa. This overexpression was particularly evident at the protein level and was highly significant for nm23-H1. In advanced cancers, overexpression was less frequently seen. Interestingly, we found the two kinds of apparently contradictory results found by other authors in separate studies: (a) an overexpression of the protein and mRNA of both isoforms in tumour tissue compared with normal mucosa, and (b) a lack of overexpression of nm23-H1 in some cases that were mainly advanced stages. Significant variations at both protein and mRNA levels were only seen for Nm23-H1. It is also worth noting that our RT-PCR technique, which allows for comparative determination of the relative expression of both mRNA in the same tissue sample, showed that higher levels of nm23-H2 than H1 mRNA were found in both tumour and normal colorectal tissue. If this were indeed the case for proteins, this could explain the higher immunohistochemical staining in normal tissues seen with the specific anti-B antiserum and the controversies about the expression of Nm23 in non-tumour tissues seen with antibodies like the antipeptide 11, which recognises both Nm23 isoforms.^{13 28 35} Taken as a whole, these data suggest that, when the overall level of Nm23 is evaluated, a minor variation in the nm23-H2 component could

Figure 4: Relative expression of nm23-H1 and H2 mRNA to β-actin mRNA detected by RT-PCR in tumour (T) compared with non-tumoral matched mucosa (N) in the same tissue specimen from some representative cases of colorectal cancer. From left to right cases 2, 7, 14, 33, 22, 20 listed in Table I and one case of metachronous liver metastasis of colic origin (LM) compared with the adjacent normal liver tissue (NL). M+ and M- indicate cases with and without metastases, respectively.

TABLE III Expression of nm23-H1 and nm23-H2 mRNA in tumour and corresponding normal tissues according to TNM staging

TNM stage	nm23-H1				nm23-H2			
	Tu	Nt	Tu/Nt	Number (%)	Tu	Nt	Tu/Nt	Number (%)
0-II	0.46 (0.11)*	0.29 (0.08)*	2.34 (0.54)**	10/14 (71)**	0.62 (0.09)	0.53 (0.10)	1.48 (0.24)	8/14 (57)
III-IV	0.39 (0.06)	0.69 (0.23)	1.00 (0.15)**	6/14 (43)**	0.66 (0.09)	0.71 (0.15)	1.21 (0.18)	8/14 (57)

Results are the mean (SEM) of the values corresponding to Nm23-H1 or -H2 expression (standardised to the expression of β -actin) in each TNM stage group. Tu: tumour tissue; Nt: normal tissue; Number: number of cases with overexpression versus total number of cases in the TNM stages group (%). *: $p < 0.01$; **: $p < 0.05$.

mask a significant variation in nm23-H1. This could explain the discrepancy in previous studies on colorectal and other cancers. Indeed, when Nm23-H1 expression was specifically analysed, a low expression of the Nm23-H1 protein in the primary tumour of colorectal carcinoma cases with distant metastases and in metastases themselves was already noted.³⁶ Yamaguchi *et al*²⁷ also reported that the expression of the Nm23-H1 protein and mRNA was lower in tumours associated with liver metastases. Moreover, if we reinterpret, using our criteria, the results of Myeroff *et al*²⁴ who reported overexpression without correlation to metastatic spread, it seems that cases without nm23-H1 mRNA overexpression were Dukes's C and D cases.

Our results point to a dual aspect of nm23 expression in tumour cells. The first one is overexpression compared with normal tissue. At first, the studies reporting an overexpression of Nm23/NDP kinases in tumours^{17 20 21} seemed contradictory to the suggestion that Nm23 is a potential metastatic repressor with decreased expression in aggressive tumours.^{5 35} This led to conflicting results, particularly in breast and colon carcinomas^{20 21 25 37} and was ascribed to the existence of the two closely related sequences, which can be independently regulated.⁶ It seems now that hyperexpression in tumoral cells compared with normal tissue

was seen in almost all human solid tumours so far studied. In our report, overexpression of both nm23-H1 and -H2 genes was mostly, but not exclusively, associated with early stage cancers and was not seen in benign cases. The overexpression could reflect an increased proliferative activity of tumoral cells³⁸ or alternatively, a yet undefined role in tumour progression. It should be noted that correlation between Nm23 expression and proliferative indexes has not emerged from published data.³⁷

The other aspect is the association between expression of nm23 and metastatic potential, which seems to be dependent upon the tissue type. Indeed, a decrease in the expression of nm23-H1 (not found with nm23-H2, when determined), associated with metastatic spread to distant organs or with shorter relapse free survival, or both, was seen in some types of human cancers, such as tumours of the breast,^{13 39 40} ovaries,¹⁸ and liver.¹⁶ Moreover, in melanomas,¹⁷ thyroid,⁴¹ colon,²⁷ and gastric carcinomas,⁴² a decreased nm23-H1 expression was seen in the metastases compared with the primary tumours. Surprisingly, in some cases such as neuroblastomas,³⁸ squamous cell lung carcinomas⁴³ and pancreatic carcinomas,⁴⁴ the overexpression seen in tumoral cells was even greater in advanced stages of the disease. Interestingly, a specific structural mutation (serine 120 to glycine) of nm23-H1 was identified in neuroblastoma associated with tumour aggressiveness.⁴⁵ This can be reminiscent of mutations in p53.

Is nm23-H1 involved in the control of metastatic potential? The question is still open. Our results show a heterogeneity in the labelling of tumour slices as also seen by others. Given that normal tissues are usually weakly or not labelled and that strong labelling was seen by immunohistochemistry in tumour acini at a variable percentage, it seems that some clones within the tumour lose the expression of nm23-H1. In fact, Yamaguchi *et al*²⁷ using immunohistochemistry, showed that liver metastases of colonic origin did not show nm23-H1 staining, while in lymph node metastases a variable and heterogeneous staining was seen. Did a loss of nm23-H1 allow tumoral cells to acquire some properties rendering them able to leave the primary tumour site or to better survive at a distant locus? Tumour progression to the metastatic stage entails multiple steps accompanied by multiple possible genetic rearrangements involving, among others, the chromosome 17 which, together with the two nm23 loci, bears several genes important for tumorigenesis.⁴⁶ Allelic

Figure 5: Relation between the Tu/Nt relative expression of nm23-H1 and H2 mRNA and the TNM pathological staging in 28 cases of colorectal carcinoma. The threshold to define overexpression of the target genes in comparison with the normal matched mucosa is shown by a dashed line. (Tu/Nt) relative expression ratio ≥ 1.2 .

deletions of *nm23*²² as well as a deletion in the coding sequence²⁶ have been reported in colorectal cancers associated with metastases although these changes were not found by others.^{24,25} The more convincing evidence, but still little reported, for a direct role of Nm23 in the control of metastatic dissemination, are the reports in which transfection of *nm23-H1* cDNA was able to reduce the metastatic potential of metastatic cell lines.^{10,11} Interestingly, the transfected cell lines exhibited an impaired ability to migrate in response to a variety of chemoattractant.⁴⁷ Very recently, the transfer of a normal chromosome 6 into the highly metastatic human melanoma cell line C8161 was reported to considerably reduce their metastatic but not tumorigenic behaviour concomitant with a noticeable increase in the level of the *nm23-H1* mRNA.⁴⁸ The mechanisms involved are as yet unknown.⁴⁹ As NDP kinases are enzymes responsible for the last step of GTP synthesis, however, they could play a part in signal transduction involving GTP binding proteins.⁵⁰ Interestingly, it has been reported that *nm23-H1* antisense oligodeoxynucleotides block the TGF- β 1 induced growth arrest of a human cell line of colic origin.⁵¹ Moreover, NDP kinase B (Nm23-H2 protein) was recently identified as a factor able to bind and to control expression of the *c-myc* and perhaps other promoters.⁵² It is conceivable that a change in the ratio of B to A monomers within the cell could regulate some gene controlling tumour progression.

The results presented here show that, in colorectal cancers, an overexpression of both *nm23-H1* and *nm23-H2* genes was seen in tumours compared with adjacent mucosa. In advanced cases, the overexpression of *nm23-H1* was significantly reduced compared with early stages. Although these results are less clear cut than in other types of cancers, this finding deserves prospective studies of human cohorts of colorectal cancer patients using selective procedure for the determination of Nm23-H1 to evaluate its prognostic value.

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- 1 Wiggers T, Arends JW, Schutte B, Volovics L, Bosman FT. A multivariate analysis of pathological prognostic indicators in large bowel cancer. *Cancer* 1988; 61: 386-95.
- 2 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988; 319: 525-32.
- 3 Laurent-Puig P, Olschwang S, Delattre O, Remvikos Y, Asselain B, Melot T, et al. Survival and acquired genetic alterations in colorectal cancer. *Gastroenterology* 1992; 102: 1136-41.
- 4 Bell SM, Scott N, Cross D, Sagar P, Lewis FA, Blair GE, et al. Prognostic value of p53 overexpression and c-ki-ras gene mutations in colorectal cancer. *Gastroenterology* 1993; 1: 57-64.
- 5 Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, et al. Evidence for a novel gene associated with low tumor metastatic potential. *J Natl Cancer Inst* 1988; 80: 200-4.
- 6 Stahl JA, Leone A, Rosengard AM, Porter L, Richter King C, Steeg PS. Identification of a second human *nm23* gene, *nm23-H2*. *Cancer Res* 1991; 51: 445-9.
- 7 Leone A, McBride OW, Weston A, Wang MG, Anglard P, Cropp CS, et al. Somatic allelic deletion of *nm23* in human cancer. *Cancer Res* 1991; 51: 2490-3.
- 8 Okada K, Urano T, Goi T, Baba H, Yamaguchi A, Furukawa K, et al. Isolation of human *nm23* genomes and analysis of loss of heterozygosity in primary colorectal carcinomas using a specific genomic probe. *Cancer Res* 1994; 54: 3979-82.
- 9 Gilles AM, Presecan E, Vonica A, Lascu I. Nucleoside diphosphate kinase from human erythrocytes. Structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme. *J Biol Chem* 1991; 266: 8784-9.
- 10 Leone A, Flatow U, Richter King C, Sandeen MA, Margoulies IMK, Liotta LA, et al. Reduced tumor incidence, metastatic potential, and cytokine responsiveness of Nm23 transfected melanoma cells. *Cell* 1991; 65: 25-35.
- 11 Leone A, Flatow U, Vanhoute K, Steeg PS. Transfection of human *nm23-H1* into the human MDA-MB-435 breast carcinoma cell line - effects on tumor metastatic potential, colonization and enzymatic activity. *Oncogene* 1993; 8: 2325-33.
- 12 Bevilacqua G, Sobel ME, Liotta LA, Steeg PS. Association of low *nm23* RNA levels in human primary infiltrating ductal breast carcinoma with lymph node involvement and other histopathological indicators of high tumor metastatic potential. *Cancer Res* 1989; 49: 5185-90.
- 13 Barnes R, Masood S, Barker E, Rosengard AM, Coggins DL, Crowell T, et al. Low Nm23 protein expression in infiltrating ductal breast carcinomas correlates with reduced patient survival. *Am J Pathol* 1991; 139: 245-9.
- 14 Hirayama R, Sawai S, Takagi Y, Mishima Y, Kimura N, Shimada N, et al. Positive relationship between expression of anti-metastatic factor (*nm23* gene product or nucleoside diphosphate kinase) and good prognosis in human breast cancer. *J Natl Cancer Inst* 1991; 83: 1249-50.
- 15 Nakayama T, Ohtsuru A, Nakao K, Shima M, Nakata K, Watanabe K, et al. Expression in human hepatocellular carcinoma of nucleoside diphosphate kinase, a homologue of the *nm23* gene product. *J Natl Cancer Inst* 1992; 84: 1349-54.
- 16 Yamaguchi A, Urano T, Goi T, Takeuchi K, Niimoto S, Nakagawara G, et al. Expression of human Nm23-H1 and Nm23-H2 proteins in hepatocellular carcinoma. *Cancer* 1994; 73: 2280-4.
- 17 Florenes VA, Aamdal S, Myklebost O, Maelandsmo GM, Bruland OS, Fodstad O. Levels of *nm23* messenger RNA in metastatic malignant melanomas: inverse correlation to disease progression. *Cancer Res* 1992; 52: 6088-91.
- 18 Mandai M, Konishi I, Koshiyama M, Mori T, Arai S, Tashiro H, et al. Expression of metastasis-related *nm23-H1* and *nm23-H2* genes in ovarian carcinomas: correlation with clinicopathology, EGFR, c-erbB-2, and c-erbB-3 genes, and sex steroid receptor expression. *Cancer Res* 1994; 54: 1825-30.
- 19 Francis B, Overmeyer J, John W, Marshall E, Haley B. Prevalence of nucleoside diphosphate kinase autophosphorylation in human colon carcinoma versus normal colon homogenates. *Mol Carcinog* 1989; 2: 168-78.
- 20 Lacombe ML, Sastre-Garau X, Lascu I, Wallet V, Thierry JP, Veron M. Overexpression of nucleoside diphosphate kinase (Nm23) in solid tumours. *Eur J Cancer* 1991; 27: 1302-7.
- 21 Haut M, Steeg PS, Willson JKV, Markowitz SD. Induction of *nm23* expression in human colonic neoplasms and equal expression in colon of high and low metastatic potential. *J Natl Cancer Inst* 1991; 83: 712-6.
- 22 Cohn KH, Wang F, De Soto-La Paix F, Solomon WB, Patterson LG, Arnold MR, et al. Association of *nm23-H1* allelic deletions with distant metastases in colorectal carcinoma. *Lancet* 1991; 338: 722-4.
- 23 Campo E, Miquel R, Jares P, Bosch F, Juan M, Leone A, et al. Prognostic significance of the loss of heterozygosity of Nm23-H1 and p53 genes in human colorectal carcinomas. *Cancer* 1994; 73: 2913-21.
- 24 Myeroff LL, Markowitz SD. Increased *nm23-H1* and *nm23-H2* messenger RNA expression and absence of mutations in colon carcinomas of low and high metastatic potential. *J Natl Cancer Inst* 1993; 85: 147-52.
- 25 Whitelaw SC, Northover JMA. The Nm23 gene and colorectal cancer [Letter]. *Gut* 1994; 35: 141.
- 26 Wang L, Patel U, Ghosh L, Chen HC, Banerjee S. Mutation in the *nm23* gene is associated with metastasis in colorectal cancer. *Cancer Res* 1993; 53: 717-20.
- 27 Yamaguchi A, Urano T, Fushida S, Furukawa K, Nishimura G, Yonemura Y, et al. Inverse association of *nm23-H1* expression by colorectal cancer with liver metastasis. *Br J Cancer* 1993; 68: 1020-4.
- 28 Royds JA, Cross SS, Silcocks PB, Scholefield JH, Rees RC, Stephenson TJ. Nm23 anti-metastatic gene product expression in colorectal carcinoma. *J Pathol* 1994; 172: 261-6.
- 29 Hermanek P, Sobin LH, ed. *UICC TNM Classification of malignant tumors*. 4th ed. Berlin: Springer-Verlag, 1992.
- 30 Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.
- 31 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.

- 32 Van Eldick LJ, Wolchok SR. Conditions for reproducible detection of calmodulin and S100 β in immunoblots. *Biochem Biophys Res Commun* 1984; 124: 752–9.
- 33 Chirgwin JM, Przybyla AE, Mac Donald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979; 18: 5294–9.
- 34 Bertrand F, Veissiere D, Hermelin B, Paul A, Capeau J, Picard J, et al. Phosphorylation of vimentin is an intermediate step in protein kinase C-mediated glycoconjugate secretion. *Am J Physiol* 1994; 266: C611–21.
- 35 Rosengard AM, Krutzsch HC, Shearn A, Biggs JR, Barker E, Margulies IMK, et al. Reduced nm23/Awd protein in tumor metastasis and aberrant Drosophila development. *Nature* 1989; 342: 177–80.
- 36 Ayhan A, Yasui W, Yokozaki H, Kitadai Y, Tahara E. Reduced expression of nm23 protein is associated with advanced tumor stage and distant metastases in human colorectal carcinomas. *Virchows Arch B Cell Pathol* 1993; 63: 213–8.
- 37 Sastre-Garau X, Lacombe ML, Jouve M, Veron M, Magdelenat H. Nucleoside diphosphate kinase/Nm23 expression in breast cancer: lack of correlation with lymph-node metastasis. *Int J Cancer* 1992; 50: 533–8.
- 38 Hailat N, Keim DR, Melhem RF, Zhu XX, Eckersckorn C, Brodeur GM, et al. High levels of p19/nm23 protein in neuroblastoma are associated with advanced stage disease and with N-myc gene amplification. *J Clin Invest* 1991; 88: 341–5.
- 39 Toulas C, Marek E, Cheutin F, Soula G, Favre G. Expression of Nm23-H1 protein is related to metastasis free survival, tumor size and oestrogen receptor level in human breast cancer. *Proceedings of the American Association for Cancer Research* 1994; 35: 224.
- 40 Tokunaga Y, Urano T, Furukawa K, Kondo H, Kanematsu T, Shiku H. Reduced expression of nm23-H1, but not of nm23-H2, is concordant with the frequency of lymph-node metastasis of human breast cancer. *Int J Cancer* 1993; 55: 66–71.
- 41 Arai T, Watanabe M, Onodera M, Yamashita T, Masunaga A, Itoyama S, et al. Reduced nm23-H1 messenger RNA expression in metastatic lymph nodes from patients with papillary carcinoma of the thyroid. *Am J Pathol* 1993; 142: 1938–44.
- 42 Kodera Y, Isobe K, Yamauchi M, Kondoh K, Kimura N, Akiyama S, et al. Expression of Nm23-H1 RNA levels in human gastric cancer tissues – a negative correlation with nodal metastasis. *Cancer* 1994; 73: 259–65.
- 43 Engel M, Theisinger B, Seib T, Seitz G, Huwer H, Zang KD, et al. High levels of MN23-H1 and NM23-H2 messenger RNA in human squamous-cell lung carcinoma are associated with poor differentiation and advanced tumour stages. *Int J Cancer* 1993; 55: 375–9.
- 44 Nakamori S, Ishikawa O, Ohigashi H, Imaoka S, Sasaki Y, Kameyama M, et al. Clinicopathological features and prognostic significance of nucleoside diphosphate kinase Nm23 gene product in human pancreatic exocrine neoplasms. *Int J Pancreatol* 1993; 14: 125–34.
- 45 Chang CL, Zhu XX, Thoraval DH, Ungar D, Rawwas J, Hora N, et al. nm23-H1 mutation in neuroblastoma. *Nature* 1994; 370: 335–6.
- 46 Backer JM, Mendola CE, Kovessi I, Fairhurst JL, O'Hara B, Eddy RL Jr, et al. Chromosomal localization and nucleoside diphosphate kinase activity of human metastasis-suppressor genes NM23-1 and NM23-2. *Oncogene* 1993; 8: 497–502.
- 47 Kantor JD, McCormick B, Steeg PS, Zetter BR. Inhibition of cell motility after nm23 transfection of human and murine tumor cells. *Cancer Res* 1993; 53: 1971–3.
- 48 Welch DR, Chen PC, Miele ME, McGary CT, Bower JM, Stanbridge EJ, et al. Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. *Oncogene* 1994; 9: 255–62.
- 49 MacDonald NJ, De La Rosa A, Benedict MA, Freije JMP, Krutzsch H, Steeg PS. A serine phosphorylation of Nm23, and not its nucleoside diphosphate kinase activity, correlates with suppression of tumor metastatic potential. *J Biol Chem* 1993; 268: 25780–9.
- 50 Lacombe ML, Jakobs KH. Nucleoside diphosphate kinases as potential new targets for control of development and cancer. *Trends Pharmacol Sci* 1992; 13: 45–8.
- 51 Hsu S, Huang F, Wang L, Banerjee S, Winawer S, Freidman E. The role of nm23 in transforming growth factor β 1-mediated adherence and growth arrest. *Cell Growth Differ* 1994; 5: 909–17.
- 52 Postel EH, Berberich SJ, Flint SJ, Ferrone CA. Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science* 1993; 261: 478–80.