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Increased levels of *nm23* H1/nucleoside diphosphate kinase A mRNA associated with adenocarcinoma of the prostate

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Summary. Overexpression of the *nm23*H1 gene has been associated with the suppression of metastasis in several solid tumors. However, in colorectal carcinoma and neuroblastoma, increased levels of *nm23* H1 nucleoside diphosphate kinase A (NDPKA) mRNA are associated with tumorigenesis. To determine the role of *nm23* H1/NDPKA in the prostate, normal and/or malignant tissue samples from 29 consecutive patients were studied. Levels of *nm23* H1/NDPKA mRNA and *nm23* H1/NDPKA mRNA protein were determined in tissue from 18 and 27 patients, respectively. In all, 16 of the 18 tumor samples expressed increased levels of *nm23* H1/NDPKA mRNA as compared with those measured in normal tissue. The level of *nm23* H1/NDPKA mRNA was > 10-fold higher in a metastatic lymph node than in normal prostate tissue. All cancer specimens and areas of prostatic intraepithelial neoplasia showed immunoreactivity with the *nm23* H1/NDPKA antibody; however, normal prostatic tissue was unreactive. These findings suggest that overexpression of the *nm23* H1/NDPKA gene occurs frequently in adenocarcinomas of the prostate and may be an early event in prostate cancer tumorigenesis.

Over the last 15 years, adenocarcinoma of the prostate has become the most common cancer of men in the United States, with the estimated incidence being 23% [2]. Prostate cancer accounts for more than 12% of cancer deaths among men, and the mortality associated with this malignancy in men is second only to that of lung cancer. This high death rate occurs despite the observation that prostate cancer is being identified at an earlier clinical stage through aggressive screening studies [3]. Therefore, biological events rather than clinical stage alone are important in the formation of metastases.

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Tumorigenicity and metastatic potential are related to a series of complex independent and linked sequential events [5]. The formation of metastases required cellular transformation, invasion through the basement membrane, cell motility, penetration of the vascular and/or lymphatic beds, angiogenesis, and proliferation at distant sites [6, 15]. Altering the ability of a cell to perform any of these activities would suppress the metastatic potential of that cell. Steeg et al. [20], in an attempt to identify the genes involved in suppressing or promoting metastasis, performed differential colony hybridization of mRNA between melanoma cell lines with low and high metastatic potentials. These experiments resulted in the discovery of a potential metastatic suppressor gene, *nm23* H1, with low levels of *nm23* H1 mRNA expression being found in highly metastatic melanoma cell lines and higher levels occurring in cell lines with low metastatic potential [20]. Additional experiments determined that *nm23* H1 is part of a gene family, and two *nm23* genes have been identified: *nm23* H1 and *nm23* H2. The gene product of *nm23* H1 is nucleoside diphosphate kinase A (NDPKA) [7], whereas that of *nm23* H2 is nucleoside diphosphate kinase B [7, 19].

Various human tumor tissues have been analyzed for expression of *nm23* H1/NDPKA mRNA. Decreased levels of *nm23* H1/NDPKA mRNA in primary infiltrating ductal breast carcinomas correlate with increased histological grade, loss of estrogen receptor status, and lymph node involvement [1, 10, 11]. However, increased levels of *nm23* H1/NDPKA mRNA have been found in most colon cancers as compared with normal colonic mucosa from the same patient [9]. Increased levels *nm23* H1/NDPKA are also associated with a poor prognosis in patients with neuroblastomas [9]. In an attempt to identify potential suppressor genes involved in adenocarcinoma of the prostate, we compared *nm23* H1/NDPKA mRNA levels and protein expression in normal and malignant prostate tissue from patients with prostate cancer and in three human prostate-cancer cell lines.

Materials and methods

Specimens

Samples of tumor and/or normal prostate tissue were obtained from 28 consecutive patients undergoing radical prostatectomy for clinically localized prostate cancer and from 1 patient undergoing bilateral pelvic lymph node dissection. All patients were treated at either the University of Kentucky Chandler Medical Center or the Lexington VA Medical Center. Informed consent was not required because all tests were performed on pathology samples. Of the 29 patients in this study, 12 had pathologically organ-confined disease (T_2), 15 had extraprostatic disease (T_3), and 2 had lymph node metastases (N_1). Neoplastic and benign tissues were identified by gross inspection and excised by a pathologist (E. R. B.). The tissue samples were snap-frozen in liquid nitrogen and stored at –70° C until they were processed for RNA extraction. A mirror image of the tissue taken for RNA analysis was fixed in formaldehyde and embedded in paraffin for immunohistochemical staining. Only samples containing 100% normal or at least 75% tumor tissue in the mirror image section were processed and included in the study. Consequently, ten samples that contained inadequate amounts of normal and/or tumor tissue for RNA extraction were excluded from Northern analysis. Prostate-specific antigen (PSA) serum levels were obtained from all patients before surgery. Pathological staging of the specimens was performed by the same pathologist (E. R. B.). The human prostate-cancer cell lines studied for relative *nm23* H1/NDPKA mRNA levels included DU145, PC3, and LNCAP (American Type Culture Collection, Rockville, Md.).

Northern-blot analysis

Intact mRNA from the cell lines was extracted using the acid guanidinium-phenol-chloroform technique and stored at –20° C [4]. Prostate specimens were homogenized in 10 ml of guanidinium solution and centrifuged at 10,000 rpm at 4° C for 10 min to remove debris. The supernatant was removed, and total RNA was isolated as described above.

In all 20 µg of total RNA was electrophoresed through a 1.5% agarose/6.6% formaldehyde gel and transferred onto GeneScreen (NEN DuPont) nylon membrane according to the manufacturer's recommendations [16]. Filters were then UV cross-linked or baked for 2 h at 80° C. The filters were prehybridized for 4 h at 62° C in 6×SSC (1×SSC = 0.15 *M* sodium chloride and 0.015 *M* sodium citrate), 1% sodium dodecyl sulfate (SDS, $NaDodSO_4$), 1 × Denhardt's, 10% dextran sulfate, and a 100-µg/ml concentration of sheared salmon-sperm DNA. The *nm23* H1/NDPKA cDNA probe was labeled by random hexamer primer with [α-^{32}P]-deoxycytidine triphosphate (dCTP; 3,000 Ci mmol), and a 1–2 × 10^6-cpm/ml concentration was added to the prehybridization solution and incubated at 62° C overnight. The filters were washed twice for 15 min each in 2 × SSC at room temperature, followed by two washes in 2 × SSC and 1% SDS at 62° C for 20 min, in 1 × SSC and 1% SDS at 62° C for 20 min, and in 0.1 × SSC at room temperature. The membranes were then exposed to Kodak XAR film for 1–4 days at –70° C. Membranes were then stripped according to the manufacturer's recommendations. Blots were rehybridized using an 18S cDNA probe to correct for RNA loading variations. The RNA level of each gene in question was determined using an Ultrascan XL laser densitometer. Normalized levels of *nm23* H1/NDPKA mRNA were obtained by dividing the *nm23* H1/NDPKA mRNA level by the 18S rRNA level.

Immunohistochemistry studies

Sections cut at 5 µm from both normal and tumor tissue samples taken from 27 of the 29 patients were analyzed for *nm23* H1/NDPKA expression. Two patients were excluded from this analysis because insufficient tissue was available for research purposes. The paraffin-coated sections were dewaxed, rehydrated, and treated for 20 min with 10% normal goat serum to prevent nonspecific binding. Sections were then incubated at room temperature for 30 min with a polyclonal anti-*nm23* H1/NDPKA antibody diluted 300-fold as previously described [18]. After the excess primary antibody was washed off, sections were incubated with a secondary goat anti-rabbit IgM antibody. The sections were then treated with diaminobenzidene tetrahydrochloride and counterstained with hematoxylin and eosin. Samples were examined by a pathologist (E. R. B.), who was blinded to other experimental results, and were graded according to the following scale: 0, no staining; +1, faint staining; +2, moderate staining; +3, strong staining; and +4, intense staining.

Statistical analysis

The normalized *nm23* H1/NDPKA mRNA levels were dichotomized in such a way that a 2-fold increase was scored as "high," whereas an increase by a factor of less than 2 was considered "low." Similarly, PSA values higher than 4 and Gleason scores of 7 and above were considered "high." Fisher's exact test was used to test for association between *nm23* H1/NDPKA mRNA levels and each of the variables (PSA, pathological stage, and Gleason score) [17]. We also conducted *t*-tests on the continuous mRNA levels comparing these levels with each of the high versus low PSA values, pathological stages, and Gleason scores obtained for each patient.

Results

Expression of nm23 H1/NDPKA mRNA

Northern analysis demonstrated expression of *nm23* H1/NDPKA mRNA in all samples containing prostate tumor or normal prostatic tissue. There was no abnormally sized transcript to suggest that the mRNA was truncated or grossly altered in form (Fig. 1). Overall, 16 of 18 tumors

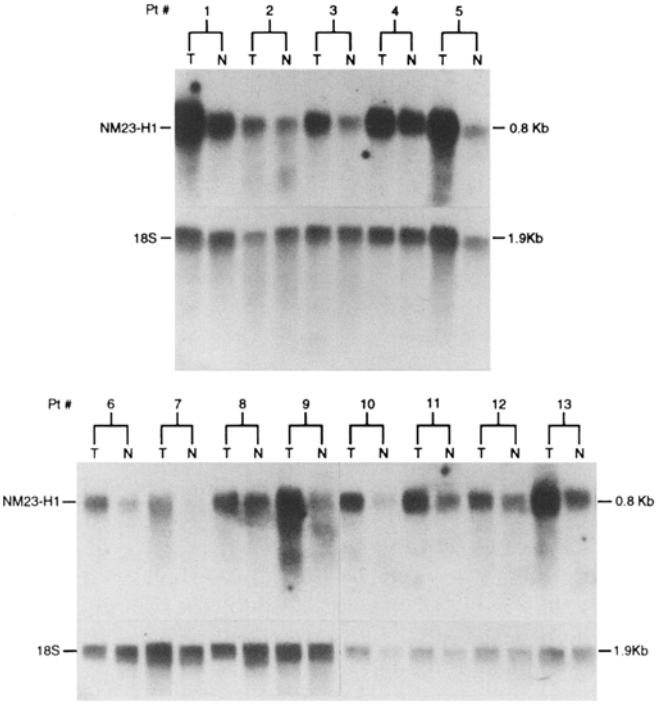
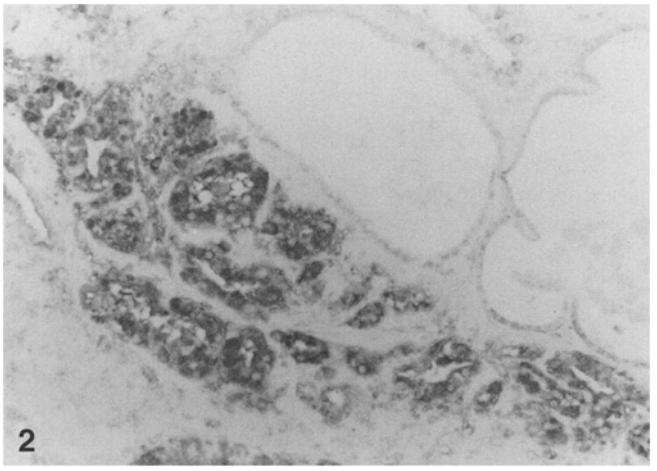


Fig. 1. Northern analysis of normal and cancerous prostate tissue hybridized with the *nm23* H1 cDNA probe. Increased *nm23* H1 mRNA levels in prostate tumors as compared with normal prostate tissue were noted in 12 of the 13 samples shown



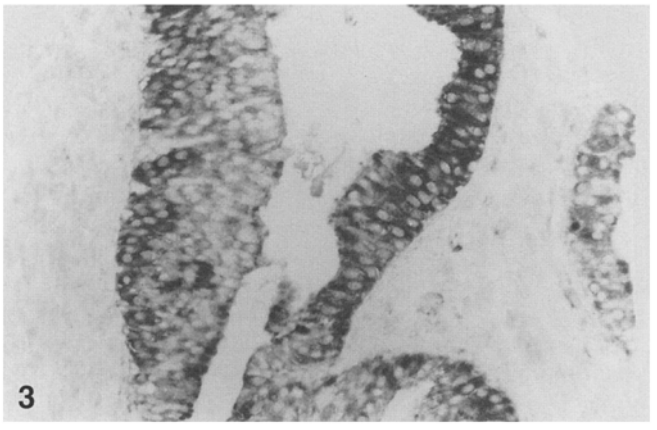


Fig. 2. Immunohistochemical staining with the NDPKA antibody revealed strong staining in the neoplastic cells and no staining in benign glands or stroma. × 200

Fig. 3. Immunohistochemical staining of prostate tissue with the NDPKA antibody. Areas of high-grade prostatic epithelial neoplasia show immunoreactivity with the NDPKA antibody. × 200

(89%) exhibited increased expression of *nm23* H1/NDPKA mRNA as compared with that seen in normal tissues from the same patient. In 61% (11/18) of the samples, the levels of *nm23* H1 mRNA were at least 2-fold as high as the levels observed in normal tissues, with a mean 5.5-fold increase and a median 4.1-fold increase being found. In an additional 5 tumor samples (28%), a 1.5- to 2-fold increase in *nm23* H1 expression was seen. No significant difference in mRNA levels was noted among the three human prostate-cancer cell lines; however, markedly elevated levels of *nm23* H1/NDPKA were present when mRNA levels in these cell lines were compared with those in normal tissue (> 10-fold). The level of *nm23* H1/NDPKA mRNA was also significantly higher (> 10-fold) in the metastatic lymph node than in normal prostate tissue.

Immunohistochemistry

Immunoreactivity to the *nm23* H1/NDPKA antibody was demonstrated in the neoplastic cells and focally in basal cells but was not seen in normal prostate epithelial cells, cells from benign prostatic hyperplasia, or stromal cells.

Table 1. Clinical, pathological, mRNA, and immunohistochemical findings in prostate cancer

Patient number[a]	Relative *nm23* mRNA levels[b]	Immuno-histo-chemical staining[c]	PSA level	Gleason grade	Patho-logical stage[d]
29	25.8	NA	48.0	9	N_1
1	12.0	4.0	20.2	8	T_3
9	10.4	4.0	8.4	6	T_3
3	9.5	1.0	6.6	8	T_2
6	5.5	1.0	8.8	8	T_3
5	4.8	4.0	17.0	7	T_3
2	4.1	NA	20.0	7	T_3
11	3.0	4.0	7.6	8	T_3
10	3.0	4.0	42.5	9	T_3
12	2.8	3.0	27.0	7	T_3
13	2.7	3.0	9.9	6	T_2
14	2.3	3.0	15.5	7	T_3
4	1.8	4.0	2.1	7	T_2
8	1.8	4.0	3.5	7	T_2
7	1.7	4.0	10.0	7	T_2
16	1.6	3.0	8.0	7	T_3
17	1.6	2.0	3.4	7	T_3
18	1.0	3.0	29.0	9	Ty_3
15	0.6	2.0	82.0	8	T_3
19	NA	3.0	6.1	5	T_2
20	NA	3.0	4.3	5	T_2
21	NA	1.0	3.0	5	T_2
22	NA	3.0	1.7	5	T_2
23	NA	3.0	12.0	6	T_3
24	NA	3.0	4.3	7	T_2
25	NA	1.0	7.4	4	T_2
26	NA	3.0	12.5	7	T_3
27	NA	3.0	2.8	5	T_2
28	NA	4.0	18.0	9	T_3N_1

NA, Tissue not available for study
[a] Patient numbers correspond to those shown in Fig. 1
[b] Relative mRNA levels represent the normalized ratio of nm23 mRNA in tumor tissue to that in normal prostate tissue
[c] Key: 0, no staining; +1, faint staining; +2, moderate staining; +3, strong staining; +4, intense staining
[d] Key: T_2, tumor confined to the prostate; T_3, extracapsular disease; N, unilateral lymph node metastases

All cancer specimens showed immunoreactivity; 22 of the 27 prostate cancers exhibited strong to intense staining in the neoplastic cells (Fig. 2, Table 1). Interestingly, areas of high-grade prostatic intraepithelial neoplasia (PIN) showed immunoreactivity, suggesting that *nm23* H1 expression occurs early in prostate cancer tumorigenesis (Fig. 3).

Relationship between disease progression and nm23 H1/NDPKA levels

PSA levels as well as tumor stage and grade, were correlated with relative expression of *nm23* H1/NDPKA mRNA (Table 2). PSA levels were elevated (> 4) in 15 samples

Table 2. Correlation of serum PSA levels, pathological stage, and Gleason score with relative *nm23* H1 mRNA expression

	Number of patients (%) [a]	*P* value
PSA level:		
≤ 4	0/ 3 (0)	0.01
> 4	11/15 (73)	
Pathological stage:		
T_2	2/ 5 (40)	0.76
T_3	9/13 (69)	
Gleason grade:		
≤ 7	6/11 (54)	0.31
> 7	5/ 7 (71)	

[a] Number of patients with relative *nm23* mRNA levels of > 2

and were normal in 3 specimens. In 12 of the 15 samples with an elevated PSA level, relative *nm23* H1/NDPKA mRNA levels were > 2; relative *nm23* H1/NDPKA levels did not exceed 2 in any of the samples (0/3) with a normal PSA level ($P = 0.013$). However, there was no statistically significant association between the level of *nm23* H1/NDPKA mRNA and the pathological stage or Gleason grade of the primary tumor. Samples from 69% of the patients with extraprostatic disease had a > 2-fold increase in expression of *nm23* H1/NDPKA mRNA as compared with 40% of the samples from patients with organ-confined tumors. Similarly, 71% of those tumors with a Gleason score of ≥ 7 and 54% of those with a Gleason score of ≥ 7 had a 2-fold increase in relative expression of *nm23* H1/NDPKA mRNA. There was no correlation between the immunohistochemical staining score and the serum PSA level, tumor stage, or Gleason score.

Discussion

We found that the levels of *nm23* H1/NDPKA mRNA in prostate tumor samples were significantly higher than the levels of *nm23* H1/NDPKA in normal prostatic tissues in 88% of patients. A > 2-fold increase in *nm23* H1/NDPKA mRNA levels was noted in 61% of the tumor samples. We confirmed that increased levels of *nm23* H1/NDPKA mRNA in prostate cancer resulted in overexpression of the *nm23* H1 protein product NDPKA. All prostate cancers and high-grade PIN stained positively with the *nm23* H1/NDPKA antibody. This antibody has been used to investigate *nm23* H1/NDPKA activity in a variety of tumors, including breast, colon, and uterine cancer. In colon and uterine metastatic lesions and primary tumors, *nm23* H1/NDPKA activity and protein levels were higher than those observed in normal tissue extracts from the same patient [14].

Interestingly, the expression of *nm23* H1/NDPKA mRNA and protein was not identical in some of our prostate samples. This discrepancy in expression of mRNA and protein may be related to the identification of both *nm23* H1 and *nm23* H2 mRNA by Northern analysis. Both *nm23* H1 and *nm23* H2 genes have a sequence homology of 88%, and both make a 0.9-kb transcript [19]. Because we used the full-length *nm23* H1 cDNA probe, mRNA from both genes would have been identified. If both genes are overexpressed, the level of *nm23* H1/NDPKA in tumors may be overrepresented as compared with the levels in normal prostate tissue. We are currently working with probes specific to *nm23* H1/NDPKA H1 and *nm23* H2 to analyze further the relative levels of expression and possible feedback interactions between the two. Additional reasons for the discrepancy include (1) half-life differences between the protein and the mRNA and (2) possible feedback interactions between the mRNA and the protein product. Nevertheless, increased expression of *nm23* H1/NDPKA mRNA and protein was seen in all stages of prostate cancer. The expression of *nm23* H1/NDPKA mRNA did not correlate with the pathological stage or grade of the primary tumor. Because increased *nm23* H1/NDPKA expression was seen in 16 of 18 tumors and the level of expression was not significantly different between the tumor samples (range, 1.5- to 12-fold increase), it is not surprising that the expression of *nm23* H1/NDPKA did not correlate with the pathological stage or grade of the tumor. This suggests that overexpression of *nm23* H1/NDPKA is an early event in prostate cancer tumorigenesis.

Our results are similar to recent reports that most prostate tumors show immunoreactivity of the *nm23* H1 antibody [12, 13]. As was true in our study, Igawa et al. [12], using an *nm23* H1 antibody different from ours, found no difference in *nm23* H1/NDPKA protein expression between T_2 and T_3 tumors. These authors did find that 71% of metastatic tumors stained intensely as compared with 19% of T_3 tumors and 23% of T_2 tumors. Although both of our samples from patients with lymph node metastases showed intense homogeneous staining, we did not find a strong correlation between immunoreactivity and the stage or grade of the primary tumor, most likely because of the difficulty involved in precisely quantitating protein levels via immunohistochemical staining. Konishi et al. [13], using a different monoclonal antibody against the *nm23* H1 protein, found positive immunostaining in 74% of clinical prostate cancers. However, in contrast to our findings and to the results reported by Igawa et al. [12], benign prostatic hyperplasia showed weakly positive, staining and metastatic prostate cancer was not immunoreactive. Our experiments finding *nm23* H1/NDPKA mRNA expression in normal and malignant prostate tissue but relatively more expression in the prostate cancer tissue suggest that *nm23* H1/NDPKA is expressed in normal prostate tissue, albeit at a lower level than in prostate cancer. The reason why Konishi et al. [13] found decreased expression in the primary tumors of patients with metastatic prostate cancer, whereas Igawa et al. [12] found increased expression in these tumors is unclear, but it may be related to the small sample size, the different antibodies used, and the different scoring system used to determine the immunohistochemical staining.

Although the *nm23* H1/NDPKA gene is generally considered a metastasis-suppressor gene in breast cancer, we found that in prostate cancer it may instead be related to tumorigenesis. The lack of correlation of *nm23* H1/NDPKA expression with known prognostic factors in prostate can-

cer (i.e., pathological stage or grade) and the overexpression seen in PIN suggest that altered expression of the *nm23* H1/NDPKA gene is an early event in prostate tumorigenesis, with continued overexpression occurring throughout tumor progression. This finding is similar to those of studies that have demonstrated increased *nm23* H1 expression in colon cancer and neuroblastoma [8, 9]. Hailat et al. [8] found that increased expression of the *nm23* H1 gene product in neuroblastoma was more reliable than N-*myc* oncogene amplification in characterizing advanced-stage tumors. Additionally, increased levels of *nm23* H1 mRNA were noted in 16 of 18 adenocarcinomas of the colon as compared with the levels seen in normal colon tissue and in 12 colon-carcinoma cell lines with varying metastatic potential [9]. In that same study, increased levels of *nm23* H1 mRNA were observed as early as in the polyp stage of colonic neoplasia, and levels remained elevated in both nonmetastatic and metastatic colon cancers [9]. In both colon carcinoma and neuroblastoma, genetic mutations were identified in the *nm23* H1/NDPKA gene. We have evaluated ten primary prostate cancers for lack of heterozygosity in the *nm23* H1/NDPKA gene loci, but none had allelic alterations at this site (data not shown).

The mechanisms and role of *nm23* H1 in tumorigenesis or suppression remain elusive. A recent study suggests that the expression of *nm23* H1/NDPKA is related to the cell cycle in prostate cancer cells [12]. Increased expression of *nm23* H1/NDPKA mRNA was seen in cells progressing though the cell cycle but was not observed in cells in the G0 phase. This finding suggests that *nm23* H1/NDPKA is a marker of proliferation rather than a metastasis-suppressor gene in prostate cancer cells. The expression of *nm23* H1/NDPKA found in a variety of tumor types suggests that the action of *nm23* H1/NDPKA may be tissue-specific.

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