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Expression of the Nucleoside Diphosphate Kinase In Human Skin Cancers : An Immunohistochemical Study

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Expression of nucleoside diphosphate(NDP) kinase, which is homologous to the nm23 gene product in a variety of species, has been found to be inversely associated with metastatic potential. However, the relationship remains controversial according to the tumor cell types and experimental system, with conflicting results from different research groups. In order to determine whether NDP kinase expression serves as a marker for metastatic potential in human skin cancer, we assessed the levels of NDP kinase expression in 9 keratoacanthomas (KAs), 26 squamous cell carcinomas (SCCs), and 25 basal cell carcinomas (BCCs) using immunohistochemistry. The expression of NDP kinase was intense in KA and SCC compared with BCC. However, the difference of NDP kinase expression between KA and SCC was not statistically significant. And there was no statistically significant difference in NDP kinase expression between SCC with metastasis and SCC without metastasis. Our results contradict the hypothesis concerning the possible role of nm23 gene as a metastatic suppressor gene in human skin cancer. The mechanism of overexpression in various tumor cell types and its biological significance in cutaneous carcinogenesis remain to be determined.

Key Words : *nm23, NDP Kinase, Keratoacanthoma, Squamous cell carcinoma, Basal cell carcinoma, Immunohistochemistry*

INTRODUCTION

The nm23 gene was originally identified by Steeg et al.(1988) by differential screening of a cDNA library with RNA from low and high metastatic clones of a murine melanoma cell line. Subsequently, a high degree of sequence homology has been reported between nm23 and nucleoside diphosphate (NDP) kinase in several species (Biggs et al., 1988 ; Kimura et al., 1990 ; Lacombe et al., 1990 ; Munoz-Dorado et al., 1990). In four rodent experimental systems, tumor cells of low metastatic potential demonstrated significantly greater nm23-RNA levels than related highly metastatic tumor cells (Steeg and Liotta, 1990). Further evidence implicating nm23 in the control of metastatic murine melanoma cells transfected with nm23 gene demonstrated a significantly reduced metastatic ability independently of tumor cell growth (Leone et al., 1991). However, not all studies associate low nm23 gene expression with metastasis. For example, in two studies of breast carcinoma, there was no significant association between lymph node

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stage and nm23 protein expression (Sastre-Garau et al., 1992; Sawan et al.,1994). Moreover, nm23 expression was associated with an increase in metastastic potential in two different murine melanoma cell lines (Morris et al., 1993).

In human squamous neoplasm, Stephenson et al.(1993) have examined the expression of nm23 product in keratoacanthoma (KA) and squamous cell carcinoma (SCC), since the former never metastasize while the latter have this potential. They found that there was no statistically significant trend in tumor staining from KA through decreasing grades of differentiation of SCC. However in this study, they considered only metastatic potential, which is directly related to degree of differentiation of SCC, rather than proven established metastasis. Furthermore, to our knowledge, studies of the product level of the nm23 gene have not yet been carried out in basal cell carcinoma(BCC), which is also known to metastasize very rarely.

We therefore performed an immunohistochemical analysis of NDP kinase expression in KA, SCC, and BCC using an affinity-purified monoclonal antibody raised in mice immunized with human NDP kinase A purified from erythrocytes to determine its correlation with metastatic potential.

MATERIALS AND METHODS

Formalin-fixed and paraffin-embedded tissue blocks from 60 biopsy specimens of skin cancer, which were well-preserved for immunohistochemical study were examined. Nine patients had KA, 26 patients had SCC (12 cases without metastasis and 14 cases with metastasis), and 25 patients had BCC. In addition, 3 normal subjects and 3 patients with psoriasis were studied as controls. From each block, one section was cut for diagnostic review while the next two sections were mounted on poly-lysine-coated slides, air-dried and heated to 6℃ for 20 minutes. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 minutes. Non-specific antibody binding was blocked with normal rabbit serum for 10 minutes. The primary antibody, an affinity-purified mouse monoclonal antibody to NDP kinase A (Novocastra, U.K.) was applied at a 1 : 200 dilution in Tris-buffered saline (TBS, pH 7.6) for 60 minutes at room temperature. Following two changes of TBS for 10 minutes the sections were covered with biotinylated rabbit anti-mouse immunoglobulin (Dako, Denmark), diluted 1 : 500, for 30 minutes. Sections were then washed in two changes of TBS for 10 minutes. This was followed by addition of the tertiary antibody, streptavidin-biotin-peroxidase complex (Dako, Denmark) applied for 30 minutes. Sections were again washed in TBS for 10 minutes and developed with diaminobenzidine tetrahydrochloride substrate (Zymed, USA). Sections were counterstained with Mayer's hematoxylin, cleaned, and mounted. We included omission and substitution controls, and positive controls with breast adenocarcinoma cases which we had previously found to give positive NDP kinase staining in each immunohistochemical batch.

Sections were reviewed by two independent observers with a conference microscope and graded as 1-4 corresponding respectively to less than 25, 50, 75, and 100% of tumor cells stained as described by Stephenson et al.(1993).

Statistical analyses were carried out using the chi-square test and Mann-Whitney's U analysis. Differences were taken as significant when P value was less than 0.05.

RESULTS

All omission and substitution controls were negative while the positive tissue controls gave uniform cytoplasmic staining. The staining pattern of NDP kinase in tumor cells was generally much greater than that in normal skin. In normal tissue samples, there was no or little NDP kinase expression with some exceptions (Fig. 1A) : sweat glands and their ducts, sebaceous glands, and some follicular epithelium often showed immunoreactivity for NDP kinase. In psoriatic lesions, we observed the overexpression of NDP kinase comparable to that of KA which showed the highest levels of expression in the set of tumors that we analysed. The 9 KA showed a mean staining grade of 2.56 and a mean median of 2.00 (Fig. 1B) ; for the 26 SCC the mean was 2.07 and the median was 2.00 (Fig. 1C, 1D) ; for the 25 BCC the mean was 1.40 and the median was 1.00 (Fig. 1E). The results are summarized in Table 1.

Staining intensities of NDP kinase of KA, SCC, and BCC were compared to find if there are any statistically significant differences associated with metastatic potential. Expression of NDP kinase was significantly less intense in BCC compared with those of KA and SCC ($p=0.0015$ and $p=0.0060$, respectively). In

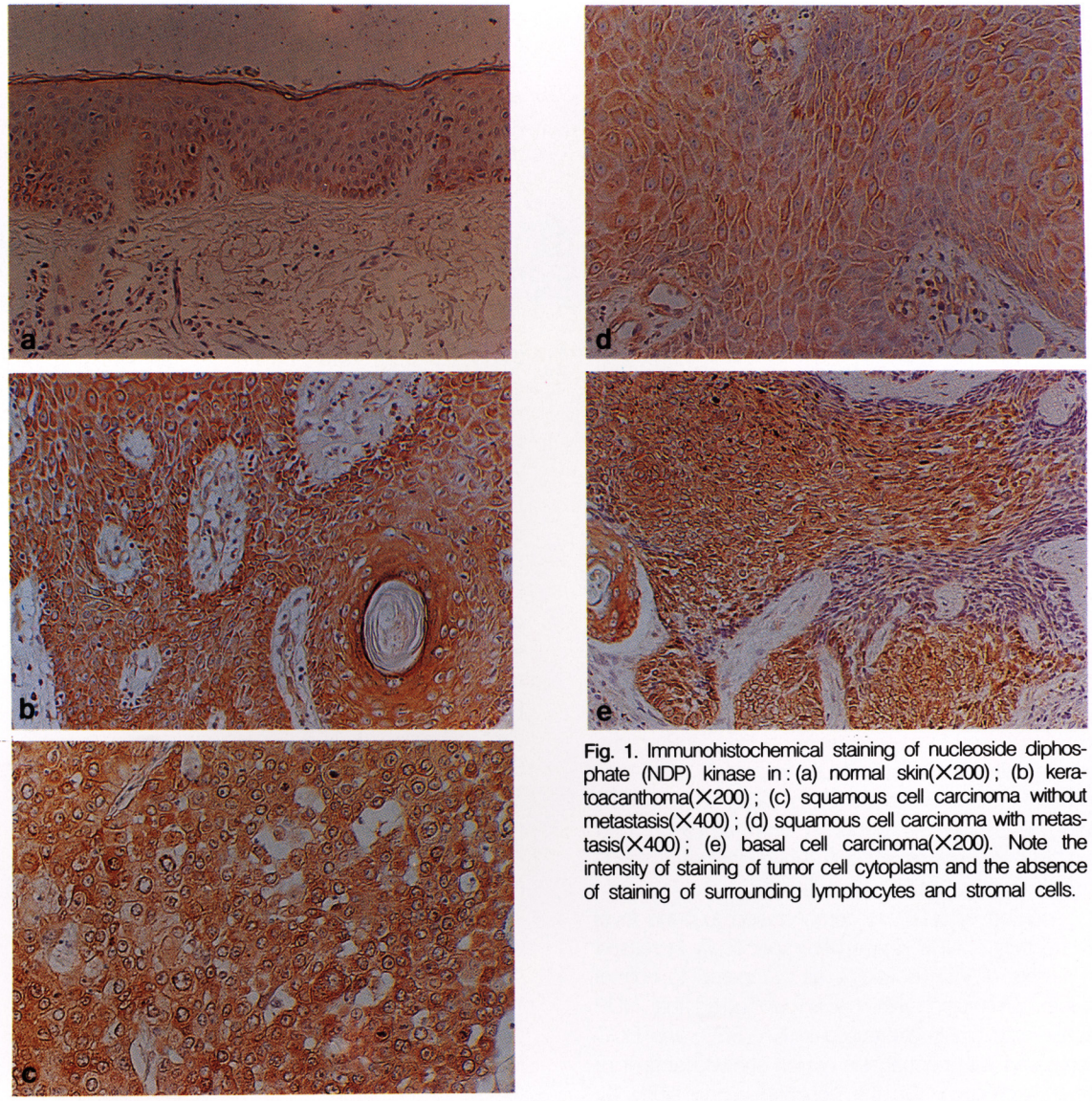

Fig. 1. Immunohistochemical staining of nucleoside diphosphate (NDP) kinase in : (a) normal skin(×200) ; (b) keratoacanthoma(×200) ; (c) squamous cell carcinoma without metastasis(×400) ; (d) squamous cell carcinoma with metastasis(×400) ; (e) basal cell carcinoma(×200). Note the intensity of staining of tumor cell cytoplasm and the absence of staining of surrounding lymphocytes and stromal cells.

Table 1. Correlation of nm23 product positivity with lesion type

Lesion type	No. of cases	Grade* of nm23 product positivity				Mean	Median
		1	2	3	4		
KA	9	1	4	2	2	2.56	2
SCC	26	8	11	4	3	2.07	2
with metastasis	12	2	6	2	2	2.33	2
without metastasis	14	6	5	2	1	1.86	2
BCC	25	17	6	2	0	1.40	1

*: Graded according to the proportion of cells stained for nm23 product : 1=up to 25% ; 2=25-50%; 3=50-75%; 4=up to 100%.
KA : keratoacanthoma ; SCC : squamous cell carcinoma ; BCC : basal cell carcinoma

contrast, the difference of NDP kinase expression between KA and SCC was not statistically significant ($p=0.2022$). The intensity of NDP kinase expression in SCC with metastasis was compared with that in SCC without metastasis : there was no significant difference between the two groups ($p=0.1920$).

DISCUSSION

The nm23 gene is located on the long arm of chromosome 17 and encoded a 17 KD polypeptide (Rosengard et al., 1989). The predicted human nm23 protein has 78% homology with the protein encoded by the developmentally regulated awd gene in *Drosophila*, mutation of which causes abnormal tissue morphology, aberrant differentiation, and necrosis (Dearolf et al., 1988 ; Rosengard et al., 1989). A gene found in the slime mould *Dictyostellium discoideum* encoding a NDP kinase is also highly homologous to both nm23 and awd (Lacombe et al., 1990 ; Wallet et al., 1990). Recently, Stahl et al.(1991) described a second human nm23 gene designated nm23-H2 possessing 88% homology with the gene originally described, thus now named nm23-H1. The identification of nm23 as an NDP kinase was recently definitively confirmed by demonstration of the identity of the primary structure of the NDP kinase A and NDP kinase B of human erythrocytes (Gilles et al., 1991) with the sequences deduced from nm23-H1 and nm23-H2 genes, respectively (Rosengard et al., 1989 ; Stahl et al., 1991).

Lacombe et al.(1991) demonstrated that the level and activity of NDP kinase were specifically increased in tumors of various origins as compared to normal tissues. Consistent with this is our finding that NDP kinase was highly expressed in KA, SCC, and BCC compared with normal skin, which showed absent or lower expression. On the contrary, in the study by Stephenson et al.(1993) where a polyclonal antibody to a synthetic nm23 peptide was used, adjacent normal epidermis and adnexal structures showed uniform intense cytoplasmic staining. Similar results have been recorded in breast cancers. In the study by Hirayama et al.(1991) and that of Sawan et al.(1994) where anti-rat and anti-human NDP kinase antibodies were utilized respectively, normal epithelium showed lower expression of NDP kinase compared with most tumors. In contrast, Barnes et al.(1991) found normal epithelium to have higher levels of expression using a polyclonal antibody to a synthetic nm 23 peptide. These conflicting results suggest that the antibody specificities and functions may differ, thus recognizing different epitopes, although the expression of NDP kinase is now known to be identical to the nm23 gene product. In this regard, the role of nm23 may not be due only to its NDP kinase activity.

Reduced expression of nm23/NDP kinase genes in tumor cells with highly metastatic characteristics has been reported in several experimental systems and certain types of human cancer such as breast cancer (Bevilacqua et al., 1989 ; Barnes et al., 1991 ; Hennessy et al., 1991 ; Hirayama et al., 1991). In the present study, in which we used an immunohistochemical technique with an antibody against purified NDP kinase A, we demonstrated that NDP kinase expression did not significantly differ between KA and SCC. Given the fact that the former never metastasize while the latter have this potential, our results contradict the hypothesis concerning the possible role of NDP kinase. These findings are in keeping with the results of Stephenson et al.(1993) who reported that there was no significant trend in tumor staining from KA through decreasing grades of differentiation of SCC. Furthermore, we could not find any statistically significant difference of NDP kinase expression between SCC without metastasis and SCC with metastasis. The reasons for the discrepancies between these results and other studies reporting an association between low expression of the nm23 gene and high metastatic potential are not clear at present. However, it should be noted that we have measured the protein level using immunohistochemical technique with monoclonal antibody against the purified protein, whereas most of the preceding studies have relied on RNA detection. It is known that the NDP kinase has higher metabolic stability and therefore, a decrease in mRNA might not be reflected at the protein level. Sawan et al.(1994) have also demonstrated the weakness of the correlation between the data of immunohistochemical analysis for NDP kinase and the nm23 mRNA levels, resulting in many mRNA-negative cases scored positive by immunohistochemistry.

Currently, the expression of nm23 mRNA evaluated by either Northern blot analysis or in situ hybridization is shown to be associated with low metastatic potential in several types of human cancer, including colon (Cohn et al., 1991) and breast carcinoma (Bevilacqua et al., 1989 ; Hennessy et al., 1991). A similar correlation between nm23 mRNA expression and tumor

aggressiveness was also found in malignant melanoma of human skin (Florenes et al., 1992). However, not all studies associate low nm23 mRNA levels with tumor metastasis. In neuroblastomas, aggressive tumors showed higher levels of nm23 mRNA with n-myc gene amplification, and it has been proposed that nm23 molecular alterations, rather than its reduced expression, can be associated with tumor aggressiveness (Hailat et al., 1991). In colonic cancer, in contrast to the evidence of nm23 allelic gene deletions in aggressive cases, Haut et al.(1991) failed to observe a similar association between nm23 mRNA and metastasis. Since the results of the studies which have measured nm23 mRNA levels as a marker for metastatic potential were also at variance, these findings are believed to be a consequence of tissue-specific phenomenon rather than the validity of the methodology of nm23 expression measurement.

The precise biologic function of nm23/NDP kinase is still unknown : as NDP kinase is important in providing nucleoside triphosphates required for RNA and DNA synthesis, an increase in NDP kinase expression and activity could correlate with hyperproliferation. Indeed, we observed the overexpression of NDP kinase in psoriasis, which might indicate a correlation with the proliferative state. Recently, Keim et al.(1992) also reported that peripheral blood lymphocytes stimulated with phytohemagglutinin showed high levels of nm23 expression. These observations would suggest involvement of nm23 in normal and malignant proliferation that is distinct from its proposed role as a tumor metastasis suppressor.

In conclusion, our data suggest that the level of NDP kinase, the product of the nm23 gene, increases in the set of skin tumors that we have analysed, but can not be considered a biological marker of metastatic potential. Further investigation is required to determine the differential role of nm23 and NDP kinase in cutaneous carcinogenesis, to characterize the mechanism of overexpression and its relation to cellular proliferation, and to elucidate the biological significance of nm23/NDP kinase in different cell types.

REFERENCES

Barnes R, Masood S, Barker E, Rosengard AM, Coggin DL, Crowell T, King CR, Porter-Jordan K, Wargotz ES, Liotta LA, Steeg PS. *Low nm23 protein expression in infiltrating ductal breast carcinomas correlates with reduced patient survival. Am J Pathol 1991 ; 139 : 245-50.*

Bevilacqua G, Sobel ME, Liotta LA, Steeg PS. *Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. Cancer Res 1989 ; 49 : 5185-90.*

Biggs J, Tripoulas N, Hersperger E, Dearolf C, Shearn A. *Analysis of the lethal interaction between the prune and killer of prune mutations of Drosophila. Genes Dev 1988 ; 2 : 1333-43.*

Cohn KH, Wang F, Desoto-LaPaix F, Solomon WB, Patterson LG, Arnold MR, Weimar J, Feldman JG, Levy AT, Leone A, Steeg PS. *Association of nm23-H1 allelic deletions with distant metastases in colorectal carcinoma. Lancet 1991 ; 338 : 722-4.*

Dearolf CR, Hersperger E, Shearn A. *Developmental consequences of awd, a cell-autonomous lethal mutation of Drosophila induced by hybrid dysgenesis. Dev Biol 1988 ; 129 : 159-68.*

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.*

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.*

Hailat N, Keim DR, Melhem RF, Zhu XX, Eckerskorn C, Brodeur GM, Reynolds CP, Seeger RC, Lottspeich F, Strahler JR, Hanash SM. *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.*

Haut M, Steeg PS, Willson JK, Markowitz SD. *Induction of nm23 gene expression in human colonic neoplasms and equal expression in colon tumors of high and low metastatic potential. J Natl Cancer Inst 1991 ; 83 : 712-6.*

Hennessy C, Henry JA, May FEB, Westley BR, Angus B, Lennard TWJ. *Expression of the antimetastatic gene nm23 in human breast cancer : an association with good prognosis. J Natl Cancer Inst 1991 ; 83 : 281-5.*

Hirayama R, Sawai S, Takagi Y, Mishima Y, Kimura N, Shimada N, Esaki Y, Kurashima C, Utsuyama M, Hirokawa K. *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*

Keim D, Hailat N, Melhem R, Zhu XX, Lascu I, Veron M, Strahler J, Hanash SM. *Proliferation-related expression of p19/nm23 nucleoside diphosphate kinase. J Clin Invest 1992 ; 89 : 919-24.*

Kimura N, Shimada N, Nomura K, Watanabe K. *Isolation and characterization of a cDNA clone encoding rat*

nucleoside diphosphate kinase. J Biol Chem 1990; 265 : 15744-9.

Lacombe ML, Sastre-Garau X, Lascu I, Vonica A, Wallet V, Thiery JP, Veron M. *Overexpression of nucleoside diphosphate kinase (Nm23) in solid tumours. Eur J Cancer 1991; 27 : 1302-7.*

Lacombe ML, Wallet V, Troll H, Veron M. *Functional cloning of a nucleoside diphosphate kinase from Dictyostellium discoideum. J Biol Chem 1990; 265 : 10012-8.*

Leone A, Flatow U, King CR, Sandeen MA, Margulies IM, Liotta LA, Steeg PS. *Reduced tumor incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells. Cell 1991; 65 : 25-35.*

Morris VL, Tuck AB, Wilson SM, Percy D, Chambers AF. *Tumor progression and metastasis in murine D2 hyperplastic alveolar nodule mammary tumor cell lines. Clin Exp Metastasis 1993; 11 : 103-12.*

Munoz-Dorado J, Inouye M, Inouye S. *Nucleoside diphosphate kinase from Myxococcus xanthus I. cloning and sequencing of the gene. J Biol Chem 1990; 265 : 2702-6.*

Rosengard AM, Krutzsch HC, Shearn A, Biggs JR, Barker E, Margulies IM, King CR, Liotta LA, Steeg PS. *Reduced Nm23/Awd protein in tumour metastasis and aberrant Drosophila development. Nature 1989; 342 : 177-80.*

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.*

Sawan A, Lascu I, Veron M, Anderson JJ, Wright C, Horne CHW, Angus B. *NDP-K/nm23 expression in human breast cancer in relation to relapse, survival, and other prognostic factors : An immunohistochemical study. J Pathol 1994; 172 : 27-34.*

Stahl JA, Leone A, Rosengard AM, Porter L, King CR, Steeg PS. *Identification of a second human nm23 gene, nm23-H2. Cancer Res 1991; 51 : 445-9.*

Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta L, Sobel ME. *Evidence for a novel gene associated with low tumor metastatic potential. J Nat Cancer Inst 1988; 80 : 200-4.*

Steeg PS, Liotta LA. *Reduced nm23 expression in tumor metastasis. Proc Am-Assoc Cancer Res, Symp 11 : Metastasis : Genetic Mechanism and Cytokine Signal Transduction 1990; 31 : 504-5.*

Stephenson TJ, Royds JA, Bleehen SS, Silcocks PB, Rees RC. *'Anti-metastatic' nm23 gene product expression in keratoacanthoma and squamous cell carcinoma. Dermatology 1993; 187 : 95-9.*

Wallet V, Mutzel R, Troll H, Barzu O, Wurster B, Veron M, Lacombe ML. *Dictyostelium nucleoside diphosphate kinase highly homologous to Nm23 and Awd proteins involved in mammalian tumor metastasis and Drosophila development. J Natl Cancer Inst 1990; 82 : 1199-202.*