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Potential contributions of antimutator activity to the metastasis suppressor function of NM23-H1

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Abstract

nm23-h1 is a well-documented metastasis suppressor gene whose mechanism(s) of action have yet to be fully elucidated. The purpose of this report is to discuss recent advances in investigating the potential role of a novel 3'–5' exonuclease activity identified recently in our laboratory, a biochemical function associated, in general, with DNA repair and replication. We have employed a site-directed mutagenesis approach to demonstrate that the 3'–5' exonuclease activity of NM23-H1 is required for its metastasis suppressor function. Consistent with a role in DNA repair, we also observe that the single yeast NM23 homolog (*YNK1*) is required for the maintenance of genomic integrity and normal kinetics of DNA repair in response to exposure to ultraviolet radiation. These results and their implications for understanding the molecular mechanisms underlying NM23-H1 functions in cancer are discussed.

Keywords

NM23; Metastasis; Metastasis suppressor; Nuclease; DNA repair; Nucleoside diphosphate kinase

Introduction

While metastasis is the major cause of cancer deaths, study of this intricate process has yet to yield significant improvements in treatment of the advanced disease. Discovery of *nm23-h1* as the first metastasis suppressor gene brought a new sense of optimism, as it became apparent that re-expression of this single gene at physiological levels in highly aggressive, malignant cell lines can reduce their metastatic potential [1]. Elucidation of the specific mechanisms underlying its metastasis suppressor activity would thus appear to hold considerable promise for developing new strategies for the treatment of metastatic cancer.

To date, three regulatory and enzymatic activities have been attributed to NM23-H1, namely, its nucleoside diphosphate kinase (NDPK), histidine kinase (hisK), and 3'–5' exonuclease (3'–5' EXO) functions. So far, the hisK activity has been implicated in metastatic suppression [2, 3], while the NDPK function was reported to be expendable for inhibiting metastatic growth [4]. To date, however, no reports exist regarding the relevance of the 3'–5' EXO activity to metastasis suppressor activity of NM23 proteins. 3'–5' EXOs are proofreading enzymes that play critical roles in DNA repair and replication, removing single nucleotides from the 3'-OH terminus of a single DNA strand [5]. A role for DNA repair activities in resisting cancer

progression is supported indirectly by reports of accelerated mutation (i.e., an enhanced mutator phenotype) in highly metastatic clones versus weakly metastatic cells from the same tumor cell population [6]. Moreover, development of metastasis depends on genetic instability of transformed cells that gives rise to populations of cells capable of metastatic spread, presumably through a Darwinian-like selection process [7,8]. Taken together, these observations suggest a novel antimutator activity that mediates metastasis suppressor activity of NM23-H1, and that the loss of this protein may accelerate the propagation of mutations critical for progression to the metastatic phenotype.

The purpose of this review is to discuss the recent research directed to this issue in our laboratory. We have conducted a comprehensive mutational analysis of the NM23-H1 molecule and have identified variants with lesions in each of its three known enzymatic functions. The ultimate goal of generating this panel has been to compare the relative contributions for each activity to metastasis suppressor function. Testing of the panel of NM23-H1 variants has been conducted by forced expression in human melanoma cell lines that lack endogenous expression of the protein, followed by measurement of metastatic potential for these cell lines in the rodent models of metastasis. In parallel, we have undertaken an analysis of the yeast NM23 homolog, *YNK1*, to address the antimutator hypothesis in a simple eukaryotic model system. These preliminary experiments have shed new light on possible novel roles for NM23 in genomic maintenance, and the importance of these functions to suppression of metastatic phenotype. Moreover, these findings suggest that in addition to its well-accepted ability to suppress motility and invasiveness of tumor cells, NM23-H1 may actually suppress mutations that are necessary for metastasis competence.

Dissecting the multiple functions of H1 using site-directed mutagenesis

While mutations have been identified previously, which interfere with the NDPK and hisK functions, much remained to be done to characterize the active site of the 3'-5' EXO. A parallel goal of this research was to identify mutants with selective lesions for each one of the three known enzymatic activities. One of our early observations was that a phenylalanine substitution at the catalytic residue histidine₁₁₈ (H₁₁₈F), which plays a critical role as the phosphate donor in the NDP and protein kinase functions of NM23-H1, did not negatively impact the 3'-5' EXO function [9]. This clear dissociation of the 3'-5' EXO activity from NDPK and hisK indicated its functional independence as well its existence as a distinct active site within the NM23-H1 molecule. These early studies also demonstrated that a K₁₂Q mutation, previously shown to disrupt the nuclease activity of the NM23-H2 isoform, not only resulted in the expected loss of the 3'-5' EXO activity in NM23-H1, but also concurrently blocked the NDPK and hisK activities. The P₉₆S mutation had been shown previously to result in reduced hisK activity in recombinant H1 preparations [10]; however, its impact on exonuclease function was never examined. This study produced similar results showing P₉₆S preparations had significantly lower hisK activity, but unexpectedly appear to reveal a coordinate reduction in NDPK activity as well. The P₉₆S substitution seems to have not affected 3'-5' EXO activity. These studies did not appear to yield a mutant, however, with a selective lesion in the 3'-5' EXO that would be suitable for addressing its role as a mediator of metastasis suppressor activity.

Based on a previous report [11], we verified that an E₅A substitution severely compromises the 3'-5' EXO function of H1. Interestingly, E₅ and K₁₂ reside at the respective N- and C-terminal borders of a β -strand (β 1) of the protein, indicating an important role of this region in the active site of the 3'-5' EXO. Importantly, our preliminary studies suggest that the hisK and NDPK activities of the E₅A variant remain essentially intact, indicating the potential utility of this mutant to address the role of the 3'-5' EXO function in metastasis suppression.

The 3'–5' EXO and NDPK activities of NM23-H1 may be required for metastasis suppressor function

From the extensive panel of mutant NM23-H1 variants generated and analyzed above, a subset has been selected for the analysis of metastasis suppressor function (for a summary of their enzymatic activities in vitro, see Table 1). Each is being transfected stably into the human melanoma cell line, 1205LU, which metastasizes to the lung with high penetrance in rodent experimental models of metastasis [12] and, importantly, is deficient in expression of both the NM23-H1 and the NM23-H2 protein isoforms. As previously described in other cell lines, forced expression of wild-type and mutant variants of NM23-H1 appears to have no significant effect on the transformed phenotype of the 1205LU cell line, as assessed in terms of proliferation rate in culture or as tumor explants in athymic nude mice. Also as expected, wild-type NM23-H1 inhibited motility and invasion capacity, although unexpectedly, all four mutants appear to exhibit normal motility- and invasion-suppressing activity, indicating that none of the three biochemical activities under investigation (NDPK, hisK, and 3'–5' EXO) appear to contribute to these phenotypes in the 1205LU melanoma cell line. Further, replication of these studies is underway, but these observations to date suggest an additional unknown function of the NM23-H1 molecule is carrying out the functions of suppressing motility and invasion, at least in the melanoma setting.

Analysis of metastasis suppressor activity of the panel of NM23-H1 variants has been analyzed using a spontaneous metastasis assay in athymic nude mice. The spontaneous metastasis model provides the most complete method for assaying metastatic potential of cells in vivo, recapitulating the major steps of the metastatic process [13]. For these studies, tumor explant cells are injected subcutaneously into the rear flank region, giving rise to a primary tumor which is excised upon reaching a critical size. Mice are euthanatized approximately 2 months later and lungs are examined for the presence of pulmonary metastases.

Consistent with previous reports in another melanoma cell line [14], forced expression of wild-type NM23-H1 decreases the incidence of pulmonary metastases, relative to 1205Lu parent and a control cell line transfected with empty vector. Preliminary studies appear to show that the K₁₂ mutation which ablates all three (NDPK, hisK, and 3'–5' EXO) enzymatic activities resulted in loss of the suppressor function. Also, exhibiting an apparent loss of suppressor function was the mutant E₅A with selectively disrupted 3'–5' EXO activity, suggesting for the first time an important role for this enzymatic activity in the mechanism of suppression. The H₁₁₈F mutant also exhibited an apparent reduction in metastasis suppressor activity, representing the first analysis of metastasis suppressor activity of this mutant to date. Clearly, additional replication will be required to solidify these conclusions, but our findings are beginning to suggest that both the 3'–5' EXO and the NDPK activities contribute to the metastasis suppressor activity of NM23-H1. We propose that as a 3'–5' EXO, NM23-H1 contributes to genetic and genomic stability and, thus, opposes the accumulation of genetic aberrations required for acquisition of the metastatic phenotype. Our demonstration of such a potential antimutator role for NM23-H1 is intriguing in light of the presumed metastatic competence of the 1205LU cell line. We currently favor a mechanism in which these cells require genetic alterations and clonal selection prior to the outgrowth of metastatic lesions in the spontaneous metastasis assay model, and these events that are suppressed by the antimutator function of NM23-H1.

NM23-H1 abrogates progression of WM793 cells toward a growth factor-independent growth phenotype

Growth factor-independence is a major hallmark of advanced metastatic cells [15]. In order to detach from the primary tumor and successfully proliferate at a secondary site, a malignant cell must develop autonomy of exogenous growth factors. The onset of growth factor independence and the metastatic phenotype are driven by genetic instability of transformed cells [8]. As implied earlier, the 3'-5' EXO and NDPK enzymatic activities predispose NM23-H1 to function as an antimutator protein, which led us to propose that the putative antimutator function may resist progression of non-metastatic, transformed cells to their metastatic forms. Under this hypothesis, NM23-H1 would not affect the early neoplastic transformation, but rather intensify the pre-existing mutator phenotype once the primary tumor has been established. In order to test this hypothesis, we have evaluated the ability of NM23-H1 to suppress the acquisition of growth factor independence, under the premise that the development of growth factor independence requires stochastic accumulation of adaptive mutations. We have engineered a panel of NM23-H1 variants with selective mutations in three enzymatic activities. The mutant proteins deficient in either NDPK, hisK, 3'-5' EXO, or all three activities have been stably overexpressed in non-metastatic, vertical growth phase melanoma cell line WM793 [12]. This cell line is almost completely devoid of endogenous NM23-H1, making it an ideal candidate for complementation studies. Prior to introduction of the WM793 panel to growth factor-free medium, the cells are adapted by propagation in serum-free and insulin-supplemented medium at high seeding densities allowing sufficient accumulation and stimulation of growth by autocrine growth factors. Following the adaptation, the cells were seeded at cloning densities (1,000 cells per well of a 6-well culture plate) in protein-free growth medium. Under these conditions, cells must adapt to proliferation in a manner independent of exogenous or secreted growth factors whose concentrations at clonal seeding density are insufficient to stimulate cell growth [12]. Over the 4-5-month selection period, forced expression of wild-type NM23-H1 effectively inhibited acquisition of the growth factor-independent phenotype relative to NM23-H1-deprived WM793 parent cell line. In contrast, disruption of NDPK/hisK (H₁₁₈F) and 3'-5' EXO (E₅A) or all three (K₁₂Q) enzymatic activities resulted in the loss of the inhibitory activity. Continuous overexpression of NM23-H1 variants was verified in WM793 cells following the growth factor-free selection. Interestingly, expression of wild-type NM23-H1 did decrease over the course of growth factor-free proliferation, indicating negative selection against NM23-H1 protein. If these results can be reproduced, they strongly suggest that the 3'-5'exonuclease, and possibly NDPK/hisK activities, may oppose genetic progression of tumor cells to the metastatic phenotype.

YNK1, the *S. cerevisiae* homolog of NM23, may participate in DNA repair

As 3'-5' exonucleases are associated with DNA repair and genomic maintenance, we have studied the contribution of the yeast NM23 homolog *YNK1* in providing genomic stability in *S. cerevisiae* [16]. *YNK1* is the only NM23 isoform expressed in this relatively simple organism, obviating the high degree of NM23 gene redundancy found in higher organisms. *S. cerevisiae* provides an excellent model for DNA repair research, as they demonstrate translational and transcriptional activities in response to DNA damage that closely mimic higher eukaryotes coupled with the fact that knockout strains for essentially all genes are available. Intriguingly, strong correlative data supports a role of *YNK1* in maintaining genomic stability. Nuclear translocation of *YNK1* dramatically increases in response to treatment with the DNA methylating agent methyl methanesulfonate (MMS) [17]. The MMS-induced increase is dependent on expression of *MEC1*, the yeast homolog of the human ATR kinase which has a pivotal role in transduction of DNA damage-induced signaling. Furthermore, the DNA repair proteins HHHF2 (histone H4 protein) and RPA135 (RNA polymerase I subunit) have also been shown to interact with *YNK1* following DNA damage. HHHF2 is required for

chromatin assembly and telomeric silencing, and it has been associated with maintaining genomic stability [18]. Prior to the initiation of our investigation, however, a direct role of *YNK1* in DNA repair had yet to be studied.

To test the hypothesis that *YNK1* has a role in DNA repair, we have used a quantitative extended-length PCR assay to determine DNA repair kinetics in the nuclear genome following DNA damage [19]. The current detection limits are 1 lesion/10⁵ nucleotides with ~ 10 ng DNA. Preliminary studies of a 9.3-kb region of the phosphofructokinase-2 gene (a key enzyme involved in glycolysis) located on the chromosome XIII suggest that ablation of *YNK1* attenuates DNA repair by up to 3 h post-UV exposure. Nucleotide excision repair is primarily implicated in repair of UV-induced DNA damage by an error-free manner and it is possible that *YNK1* could be involved in the NER pathway by functioning as a novel 3'–5' EXO to remove bulky lesions such as pyrimidine dimers and 6-4 photoproducts. Efforts are being directed to identify the specific DNA repair pathways which may require the participation of *YNK1* and, ultimately, NM23-H1 in human cells.

To determine whether the deficit in DNA repair impacts upon the mutator phenotype in *S. cerevisiae*, mutation rates were measured using a conventional *CAN1* forward mutation assay following DNA damage. Mutation rates appear to be significantly increased in the *YNK1*Δ strain following treatment with either UV radiation or MMS. Mutation spectral analysis of the *CAN1* gene from the *YNK1*Δ strain after UV treatment also appears to indicate significantly increased rates of base substitution and frameshift mutations. Homonucleotide tracts of T's seemed particularly vulnerable to mutations, implicating defective translesional repair. Studies underlying the biochemical processes contributing to these observations in the mutant yeast are currently underway and are potentially relevant to understand the development of the mutator phenotype and its role in metastasis.

Conclusion

Evidence for a role of NM23-H1 in genomic maintenance has been growing, with recent studies in our laboratory showing that both NDPK and 3'–5' EXO activities appear to play important parts in establishing an antimutator phenotype. However, it should be noted that the NM23-H1 molecule is multifunctional and, thus, multiple pathways may be impacted by loss of NM23 that could be relevant to metastatic suppression. One of the most novel aspects of this model is that NM23-H1 may exert antimetastatic activity at a much earlier time during malignant progression than previously appreciated. Figure 1 outlines a model in which loss of NM23-H1 may occur in the primary melanoma, representing a metastasis progenitor cell with increased potential for mutation. Consistent with this model is the finding that a cell line derived from an aggressive but non-metastatic melanoma (WM793) exhibits almost no NM23-H1 expression, a characteristic documented previously in only metastatic cell lines and lesions. Moreover, the WM793 cell line is highly adaptable to proliferation in a growth factor-free environment, and the adaptation appears to be impaired by forced expression of NM23-H1. Importantly, this impairment appears to be dependent upon the 3'–5' EXO and NDPK activities, which is consistent with the antimutator hypothesis. Controversy has emerged recently regarding a model describing a Darwinian clonal selection for metastatic competence (i.e., mutation-driven) versus a non-mutational, adaptive mechanism for growth at the distant site (for reviews, see reference[20,21]). While both models have significant merit and are not necessarily mutually exclusive, our discovery of antimutator activity for the NM23-H1 provides significant support for the former.

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Fig. 1. NM23-H1 may suppress not only the metastatic phenotype in melanoma cells, but also progression to metastasis competence in early stages of malignant progression. Displayed within filled ovals are various functions attributed to the NM23-H1 molecule, with likely enzymatic mediators indicated adjacent to each. RGP radial growth phase, VGP vertical growth phase

Table 1

Summary of enzymatic activities for wild-type and mutant variants of NM23-H1 used for stable transfection and analysis of metastasis suppressor function

H1 variant	NDPK	hisK	EXO
Wild type	+++	+++	+++
E ₅ A	+++	++	–
K ₁₂ Q	–	–	–
P ₉₆ S	+	+	+++
H ₁₁₈ F	–	–	+++