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The Nm23-H1 metastasis suppressor as a translational target

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ABSTRACT

Nm23 was the first of what has become a field of over 20 known metastasis suppressor genes (MSGs). Since the discovery of Nm23 in 1988, a variety of mechanisms have been attributed to its activity, including a histidine kinase activity, binding of other proteins to regulate metastatic formation, and altered gene expression downstream of Nm23. Here, we will review current efforts to translate the previous work done on this MSG into the clinic, including high-dose medroxyprogesterone acetate (MPA), which has been shown to upregulate Nm23 expression. In addition, we will detail a new potential target downstream of Nm23. LPA1 is one of a group of known cell surface receptors for lysophosphatidic acid (LPA), which has been shown to be inversely correlated with Nm23 expression. A specific LPA1 antagonist could conceivably mimic the effects of Nm23 by downregulating the activity of the LPA1 pathway, which would be of considerable interest for potential clinical use.

1. Introduction

Metastasis, the process in which cancer cells leave the primary tumour and spread to other tissues and organs to form new tumours, is the most common cause of cancer mortality. In order to increase the survival of patients, it is necessary to develop more effective methods for treating established metastatic disease and, ultimately, to develop methods that prevent the establishment of metastasis altogether. The spread of single cancer cells into the vasculature or to a secondary site does not by itself constitute metastasis.[1] Rather, the development of clinically detectable metastases requires that these cells complete a series of well-defined steps. To begin with, tumour cells must break away from the primary tumour, migrate through the extracellular matrix, and intravasate into the circulatory or lymphatic systems. There, the cells must resist anoikis, survive transport, and evade immune detection before arresting or adhering in major capillary beds. Although extravasation may or may not ensue, proliferation and colonisation of these cells in the secondary parenchyma follow. These steps are together referred to as the metastatic cascade and completion of each step is necessary for metastatic development.[2] At the time of diagnosis, it is believed that only 6% of breast cancer patients will present with clinically detectable metastatic disease. Thus, the metastatic cascade is incomplete in the majority of patients, providing a valuable window of opportunity for clinicians to exploit. It is within this window, prior to colonisation and the growth of large metastasis, that the possibility exists to significantly impact patient survival.

Targets of increasing interest for this are the metastasis suppressor genes (MSGs). These are genes that, by definition, do not affect the growth of the primary tumour, but significantly inhibit the process of metastasis and reduce the formation of metastatic foci. Twenty-three such genes have now been described in the literature and collectively make up the metastasis suppressor gene family.[3] These genes, path-

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ways and downstream molecules have become the focus of significant research during the past decade.

2. Nm23-H1

The first metastasis suppressor gene, *nm23*, was identified in 1988 by differential colony hybridisation.[4] It was first discovered by injecting seven cell lines derived from a single K-1735 murine melanoma into syngeneic and nude mice. All the cell lines formed primary tumours, but varied widely in the number of metastases present at the experimental endpoint. Differential gene expression studies identified a candidate cDNA, *nm23*, the expression of which was downregulated in five highly metastatic cell lines as compared with two related, less metastatic cell lines. Ectopic expression of *nm23* suppressed metastasis without altering primary tumour growth. These findings provided the evidence that the expression of specific genes is reduced in tumour cells that have acquired the ability to form metastases and the reintroduction of such genes can suppress the metastatic phenotype.

Therapeutic approaches to restore the anti-metastatic function of *Nm23-H1* (the first in the family of eight *Nm23* human homologues) have been attempted using a range of different strategies including *Nm23-H1* promoter activation by medroxyprogesterone acetate (MPA) treatment, activation of downstream gene targets, and gene therapy, all of which will be reviewed later in this article.

3. Activities of Nm23-H1

Nm23-H1 has a variety of validated molecular activities, with at least some playing important roles in regulating its ability to inhibit metastasis.[3] The first of these activities is that of a nucleoside diphosphate kinase (NDPK) that reversibly catalyses the phosphorylation of nucleoside 5′-diphosphates to triphosphates via autophosphorylation of an internal histidine (H118). Although this activity is well characterised, there have been no reported correlations between the NDPK activity of Nm23-H1 and its metastasis-suppressive effects.

The phosphohistidine of Nm23-H1 also participates as a histidine protein kinase. Histidine kinases are best studied in bacteria and lower eukaryotes, where they function in two-component signal transduction systems, transferring a phosphate from a sensor protein to a conserved aspartate residue on a response-regulator protein in reaction to an external stimulus.[5,6] In mammalian cells histidine kinases are poorly studied because of the acid lability of the histidine phosphate, which renders it undetectable under many common experimental conditions. Despite this, there are three known substrates for Nm23-H1 as a histidine kinase.

The first is ATP-citrate lyase, the primary enzyme that catalyses the production of cytosolic acetyl-CoA.[7] Acetyl-CoA is used in multiple biosynthetic pathways including lipogenesis and cholesterogenesis. Nm23-H1 phosphorylates a histidine residue at the catalytic site of ATP-citrate lyase, although the effects of this phosphotransfer remain unknown.[7] The second substrate of the histidine kinase activity of Nm23-H1 is aldolase C, an enzyme found primarily in the brain that is critical in glycolysis.[8] Nm23-H1 phosphorylates aldolase C on aspartate 319, although once again the biological ramifications of this phosphorylation are not understood. The last known histidine kinase substrate for Nm23-H1 is the kinase suppressor of Ras (KSR). KSR is a scaffold protein for the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway.[9,10] To date no clinically relevant therapeutic targets have been developed from these known substrates of Nm23-H1.

4. Medroxyprogesterone acetate

Recently, compounds capable of upregulating MSGs in cancer cells have been reported. One of these is MPA, which has been shown to elevate Nm23 expression at high doses in MDA-MB-231 and MDA-MB-435 human breast carcinoma cell lines. MPA was identified by analysis of the *Nm23-H1* promoter, which revealed a 248-bp region regulating reporter activity.[11] This region contained a cassette for transcription factor binding sites present in the mouse mammary tumour virus-long terminal repeat. This cassette of transcription factors is regulated by glucocorticoid response elements, presenting a potential target for the upregulation of Nm23. MPA binds progesterone, androgen and glucocorticoid receptors. At high doses, MPA was found to upregulate Nm23 expression in progesterone receptor-negative, glucocorticoid receptor-positive metastatic MDA-MB-231 and MDA-MB-435 breast carcinoma cell lines *in vitro*.[11] Exposure to high-dose MPA led to decreased anchorage-independent colonisation, which was abrogated once cells were transfected with antisense *Nm23-H1*, proving that MPA was functioning by elevating Nm23 levels.

To test the hypothesis that the elevation of Nm23 by MPA would lead to decreased metastatic colonisation *in vivo*, we set up the following experiment. MDA-MB-231 breast cancer cells were injected into immunocompromised mice and 4 weeks later, at which point micrometastasis was present, the mice were randomised to vehicle or MPA. MPA dosing reduced the number of gross pulmonary metastases in these mice from 33% to 46% and reduced both incidence and size of the metastases that formed. Limited side-effects recorded, including weight gain, while no change in bone density, mammary tissue histology or the lean to fat tissue ratio were found.[12]

Based on these data, as well as on previous Nm23-H1 studies, a Phase II trial has been initiated to test this new potential application of high-dose MPA (Kathy D. Miller, PI, Indiana University). The primary objective is to determine the clinical benefit of MPA monotherapy and MPA + low-dose oral cyclophosphamide and methotrexate ('metronomic therapy', IdoCM) in postmenopausal patients with refractory hormone receptor-negative metastatic breast cancer. A starting daily oral dose of 1 g MPA will be administered and increased to 1.5 g if serum concentrations are <50 ng/ml. In a second cohort, 'metronomic' IdoCM will be administered based on its reported anti-angiogenic activity.[13] Preclinical studies suggested greater activity when metronomic chemotherapy is combined with a second anti-angiogenic agent.[14,15]

The trial includes several pharmacodynamic measurements to test whether Nm23 levels are affected in the trial. The formalin-fixed, paraffin-embedded block from the patients' primary tumours will be stained for Nm23 expression. In addition, multiple skin biopsies will be obtained from consenting patients in order to determine whether overall Nm23-H1 expression levels have been elevated by MPA. As MPA has a potential anti-angiogenic effect, the plasma thrombospondin and plasminogen activator inhibitor-1 (PAI-1) levels will also be studied during the trial. It is hoped that these tissue samples, as well as plasma from patients, will allow for a thorough understanding of the effects of MPA on Nm23-H1 levels in patients.

Although MPA has fallen out of use for the treatment of breast cancer in favour of tamoxifen, it is still used for treating endometrial cancer. A recent multicentre study enrolled 45 young patients with endometrial cancer or atypical hyperplasia, using a daily oral dose of 600 mg MPA and low-dose aspirin with the objective of preserving fertility. Either oestrogen–progestin therapy or fertility treatment was provided to responders following MPA therapy. The primary end-point, pathological complete response, was obtained in 55% of endometrial carcinoma cases and 82% of atypical hyperplasia cases. Two of the enrolled patients had grade three body weight gains and one patient had grade three liver dysfunction. During a 3-year follow-up period, 12 pregnancies and seven normal deliveries were achieved. Fourteen recurrences were found in 30 patients during follow-up of between seven and 36 months.[16] Similar results were reported in a second trial using 400 mg daily.[17] These data provide additional supporting evidence for a beneficial effect of high-dose MPA.

Currently, the most difficult issue facing the development of potential anti-metastatic agents is the clinical testing of these compounds. Potential therapeutics enter a dose finding Phase I trial in which metastatic patients in whom all approved therapies have failed have been enrolled. The potential therapeutics are then required to reduce the size of a metastatic tumour for a partial or complete response, or to maintain their current size for stable disease. It is difficult to believe that an agent targeting a step in the metastatic process will be capable of shrinking already overtly established metastases. These agents therefore often 'fail' in early clinical testing and are never advanced to a neo-adjuvant setting in which they may actually function. Newer trial designs with meaningful pharmacodynamic end-points and even new model systems to analyse these targets prior to clinical testing are needed. This is particularly true in attempting to target pathways involved in metastasis suppressor gene functions. These targets appear to be involved mostly in the early stages of metastasis, prior to the formation of clinically detectable lesions.

5. Gene therapy

Since relative levels of Nm23 appear to be important for the regulation of breast cancer metastasis, *Nm23* has been a logical target for attempts at gene therapy. There have been technical issues with re-expressing this gene in distant metastases that have only recently begun to be overcome.

Using an adeno-associated virus (AAV), Li et al. showed that in an ovarian cancer model, the transfer of *Nm23-H1* by intraperitoneal injection increased Nm23 expression in the orthotopically implanted ovarian cells compared with lac-z control vectors.[18] This increased expression of Nm23-H1 led to a 35-d increase in survival time of the mice receiving the AAV-transferred *Nm23-H1* gene. Importantly, the exogenous gene was expressed in more than 95% of the tumour cells in nude mice, which led to the 60% reduction in the number of animals developing liver metastasis. This high efficiency gene transfer, accomplished by two or three injections of AAV *Nm23-H1*, will be a necessity for any possible clinical treatment.

More recently, attempts have been made to use nanovectors to deliver the *Nm23* gene into target tissues.[19] One non-viral vector recently described is a poly-L-lysine-modified iron oxide nanoparticle (IONP-PLL), which has an average diameter of 58 nm and has apparently high transfection efficiency *in vitro* and *in vivo*. IONP-PLL bound with target DNA that have been intravenously injected into mice were found to be distributed to multiple organs, including brain, spleen, kidneys and the lungs. These results indicate that this vector may prove to be useful for systemic gene therapy. IONP-PLL with incorporated *Nm23* plasmids were injected into mice with B16F10 melanoma cells via the tail vein. Staining of subsequently formed lung metastases showed increased Nm23 expression in both tumour and stromal cells. Treated mice had significantly fewer metastases than mice treated with vector control IONP-PLL particles or naked *Nm23-H1* plasmids. Interestingly, the gene therapy combined with additional chemotherapy, cyclophosphamide, led to significantly longer survival times and even greater suppression of metastatic growth, indicating that re-expression of *Nm23* may at least provide an additive effect with traditional chemotherapeutics.

While these approaches hold promise, they are still a long way from becoming a clinical candidate. Early work with gene therapy was sometimes overly optimistic and faced significant technical hurdles that were poorly understood at the time.

6. Lysophosphatidic acid

Another growing target of interest is LPA1 (also known as EDG2), one of the receptors for lysophosphatidic acid (LPA). In previous studies we have shown that LPA1 levels are inversely correlated with Nm23 levels *in vitro*, *in vivo*, and in patient tumour samples.[20,21] Microarray analysis was performed on control transfected and *Nm23-H1*-transfected (C100 and H1-177, respectively) MDA-MB-435 cells to profile differential mRNA expression. In order to cull the resulting long list of differentially expressed genes, two additional transfectants with mutant forms of *Nm23* (P96S and S120G), which no longer possess the migratory inhibitory effects of wild-type Nm23, were also profiled.[22] The resulting genes, which were coordinately downregulated in H1-177 cells compared with C100 cells, and upregulated in at least one of the two *Nm23-H1* mutant transfectants, were then selected. These included growth factors (*CTGF*, *PTN*), receptor tyrosine kinases (*c-MET*), adhesion molecules (*L1CAM*) and G-protein coupled receptors

(*EDG2/LPA1*).[20,21] The expression differences were validated at the protein level in control and *Nm23-H1* transfectants of the MDA-MB-231 breast carcinoma cell line and subsequently in a panel of unrelated human breast cancer cell lines. To determine which of these genes functionally contributed to the suppression of motility by Nm23-H1, we asked if the restoration of their expression in *Nm23-H1*-transfected cells could restore motility to serum *in vitro*. Cells were transiently transfected with *CTGF*, *LPA1*, *MMP2*, *c-MET*, *L1CAM*, *PTN*, *FZD*, *SMO* or a vector control and their motility was determined in Boyden chamber assays. Only the re-expression of LPA1 was able to restore motility to NM23-H1-expressing cells *in vitro*. A close homologue, LPA2/EDG4, which is also activated by LPA, was tested in the same model system and did not display the same activity, indicating a selectivity in this family of receptors.

LPA1 expression levels were reduced in tumour cell lines expressing wild-type Nm23-H1, but not the two mutations incapable of suppressing tumour cell motility. In addition to this, *LPA1* levels were inversely correlated with *Nm23-H1* levels in two published microarray cohorts of human breast carcinomas.[23,24] Finally, an inverse correlation was also observed by immunohistochemical staining for EDG2 and Nm23-M1 in hepatocellular carcinoma tissues from wild-type and Nm23-M1 null mice,[20] indicating that this inverse relationship held true in multiple cell types and in clinical tissues.

We then asked whether EDG2 re-expression in the H1-177 cells would overcome its metastasis-suppressive phenotype *in vivo*. Stable transfectants of Nm23-H1 cells expressing either a vector or LPA1 were created. When injected into the mammary fat pads of nude mice, primary tumour size did not vary significantly between transfectants.[21] However, the incidence of pulmonary metastases was 51.9% in mice with the Nm23-H1 vector clones, and 90.4% for the Nm23-H1/LPA1 clones ($p = 0.000024$). This restoration of metastatic ability was comparable to that of the low Nm23-H1-expressing parental cell line, with 89.3% incidence. The median number of pulmonary metastases was two (range 0–26) in the Nm23-H1 vector lines and significantly increased to four (range 0–68) in the Nm23-H1/LPA1 clones ($p = 0.0035$); however, this number did not equal that of the control (10 in experiment 1, 4.5 in experiment 2).[21] The data indicate that LPA1 re-expression in Nm23-H1-expressing breast cancer cells can overcome many of the aspects of Nm23-H1 inhibition of *in vivo* metastasis.

This is of considerable interest as LPA1 is a cell surface receptor and therefore considered an easily druggable target. In fact, a series of LPA receptor inhibitors has been developed with varying degrees of specificity toward LPA1. A recent study by Boucharaba et al. has demonstrated the potential of one of these inhibitors, Ki16425.[25] They previously demonstrated that LPA is co-opted by breast and ovarian cancer cells as a tumour mitogen and inducer of Il-6 and Il-8, both promote the progression of bone metastases. Using Ki16425, an inhibitor of LPA1 and LPA3 receptors, they showed a significant decrease (90%) in osteolytic lesions of treated mice. Additionally, they showed a substantial decrease in the recruitment of mature osteoclasts to the bone–tumour interface in treated mice, as well as a decrease in the proliferation index of tumour cells (80% decrease in Ki67 staining). Additional studies will be required to verify the effects of specific LPA1 inhibition on metastatic progression. These results show promise for the development of specific LPA1 receptor antagonists, which could eventually become clinical therapeutic candidates.

7. Concluding remarks

The field of metastasis suppressor genes has grown dramatically throughout the past several decades. Recent insights into the ability of Nm23 to inhibit metastasis have shed light on new avenues of research and possible drug targets. The ability of LPA1 to overcome the metastasis suppressor ability of Nm23, both *in vitro* and *in vivo*, makes it an attractive therapeutic target. Defining the mechanism by which Nm23-H1 transcriptionally downregulates LPA1 remains a lingering question and an area of ongoing study that will need to be addressed. The advances in these fields of research, as well as improved understanding of the mechanisms by which Nm23-H1 function, lend hope to the possibility of future clinical implications for this metastasis suppressor gene.

Conflict of interest statement

None declared.

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