Am. J. Hum. Genet. 63:6–10, 1998

CANCER GENETICS '98
Nm23-H1: Genetic Alterations and Expression Patterns in Tumor Metastasis

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Tumorigenesis is a result of multiple mutational "assaults" on a cell that induce genetic instability and ultimately cause misregulation of components that are essential for establishing a cellular-growth balance. The genetic "hits" include the misregulation of gene transcription—which affects growth-factor production and expression of cytoplasmic and nuclear-signaling components—as well as the loss or inactivation of DNA repair enzymes and the abnormal regulation of growth-factor receptors. As a result of these multiple defects, the balance between cell growth and cell death is skewed toward immortalization. Additional changes include the acquisition of anchorage independence and the loss of contact inhibition in cellular growth. Thus, the cells then can proliferate continually, growing one on top of another to produce a mass.

Further progression of the tumor cells from a benign mass to a malignant tumor occurs when a single cell obtains further genetic alterations affecting factors responsible for keeping the cell static. For example, some of the known oncogenes (such as *RAS* and *SRC*) that cause cell transformation also induce metastatic competence (see Narod 1998 [in this issue]). Similarly, production or activation of several serine, metalloaspartyl, and thiol proteases supports the motility and invasion of tumor cells through degradation of extracellular matrix barriers (reviewed in Liotta et al. 1991; Steeg 1991). Cells that accumulate such alterations then can invade the surrounding tissue, an event that marks the beginning of metastasis.

Metastasis is a principal cause of death of patients diagnosed with solid malignant tumors. The metastatic cascade has many facets linked in sequential steps: cells must leave the primary tumor, invade the surrounding tissue, enter the circulatory or lymphatic system, extravasate into a distant organ, stimulate angiogenesis, and finally proliferate and flourish as a secondary colony (reviewed in Liotta et al. 1991). Although tumor metastasis must be measured in vivo by injecting tumor cells into immunocompromised animals, aspects of the process can be observed by means of a variety of cell-culture techniques, such as assays for cell attachment, motility, protease activity, invasion, colonization, and angiogenesis (reviewed in Welch 1997).

As with other complex cascades, metastasis is controlled, in part, through the induction and repression of genes. To identify differentially expressed genes that might be essential for metastasis, we performed differential colony hybridization on related murine melanoma cells. From this study, we identified Nm23-H1 as an mRNA species that accumulates at levels 10-fold higher in cells with low metastatic activity than in their highly metastatic counterparts, suggesting a role for this gene in the metastatic progression (Steeg et al. 1988). More-direct evidence that Nm23-H1 participates in—and possibly regulates—this process required a series of expression and transfection experiments. In multiple cohorts of tumors, low Nm23-H1 protein and mRNA expression in the tumor specimens correlated with poor clinical prognosis and survival, lymph node infiltration, and histopathological indicators of high metastatic potential. This occurred in a variety of tumor types, including breast, melanoma, gastric, and ovarian carcinomas (reviewed in De La Rosa et al. 1995). No mutations within the coding region of Nm23-H1 were reported, suggesting that expression of Nm23-H1, rather than its intrinsic activity, was the limiting factor in metastatic cells. Experiments in which Nm23-H1 cDNA was transfected into melanoma and breast carcinoma cell lines confirmed this conclusion. When transfectants were injected subcutaneously or transplanted into the mammary fat pads of nude mice, both the parental carcinoma cells, which expressed Nm23-H1 at low levels, and the transfected cells yielded primary tumors, but clones producing exogenous Nm23-H1 had a reduced potential for metastasis (Leone et al. 1991*a*, 1993*a*). In addition, Nm23-H1 overexpression specifically inhibited in vitro motility

Received May 13, 1998; accepted for publication May 15, 1998; electronically published June 5, 1998.

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0002-9297/98/6301-0003$02.00

and colonization of metastatic cells, two key events in the completion of the metastatic pathway (Leone et al. 1991*a,* 1993*a*). Thus, Nm23-H1 is not a tumor suppressor but is a metastasis suppressor.

Mutations of Nm23

Whereas loss of Nm23-H1 expression predicts aggressive metastatic growth of melanoma and mammary carcinomas, aggressive neuroblastoma tumors at stages III and IV exhibit elevated levels of Nm23-H1 RNA, a predictor of poor patient survival (Leone et al. 1993*b*). SSCP analysis of primary neuroblastoma tumors at different disease stages versus normal tissue identified a lesion in the coding region of Nm23-H1 (Leone et al. 1993*b;* Chang et al. 1994). In several instances, this point mutation, S120G, occurred as a somatic change that is associated with advanced neuroblastoma but not with earlier stage disease or with other kinds of tumors. In one case, this same allele was found in the constitutional tissue of a neuroblastoma patient and the patient's father (Chang et al. 1994). Enhanced Nm23-H1 expression and amplification of one of the genomic alleles was observed only in advanced-stage cases (Leone et al. 1993*b*). In general, however, cancers containing overexpressed, mutated Nm23-H1 protein are rare events, whereas loss of expression of Nm23-H1 is a common feature of aggressive, poorly differentiated tumors.

Genetics of *NME1*

NME1, the gene that encodes Nm23-H1, has been mapped to 17q.21, a locus that is linked to familial early-onset breast and ovarian cancer (Spurr et al. 1993) and to the breast cancer suppressor gene *BRCA1*. RFLP analysis of human chromosomal DNA in the normal population identified two alleles, indicating the existence of the two polymorphic variants of the gene (Leone et al. 1991*b;* Yague et al. 1991). Two independent investigations for allelic deletions of *NME1* in matched breast normal and tumor tissues found that 20%–64% of heterozygous breast tumors exhibited deletion of one of two polymorphic *NME1* alleles (Leone et al. 1991*b;* Futreal et al. 1992; Watatani et al. 1993). Allelic loss of *NME1* also was reported for 42% of lung adenomas and 20% of kidney carcinomas but was not detected in squamous cell carcinomas, large cell carcinomas, and other adenomas (Leone et al. 1991*b*). No association between this loss of heterozygosity (LOH) and poor prognosis among breast cancer survivors was detected (Cropp et al. 1994). Most likely, deletions that lead to LOH for the *BRCA1* gene account for the inconsistent deletion of *NME1*. Nevertheless, reduced Nm23-H1 protein expression, which was not predicted by LOH, was associated with a poor prognosis (Cropp et al. 1994). Thus, we propose that reduction of Nm23-H1 RNA and protein expression may result from RNA instability and/or an aberration of transcription regulation, independent of LOH, and that these regulatory events might be manipulated to restore normal endogenous Nm23-H1 levels in highly metastatic cells. As a step toward understanding the mechanism(s) of differential Nm23-H1 expression occurring between low and high metastatic cell types, we have cloned a 2.1-kb promoter region of *NME1* (De La Rosa et al. 1996) and have begun to analyze the transcriptional regulation of the gene.

Nm23-H1 in Differentiation and Development

Is the ability of Nm23-H1 to suppress both cell motility and colonization related to events that occur during development? The first evidence to link Nm23-H1 to normal development was reported for the *Drosophila* homologue of *NME1,* abnormal wing discs (*awd*) (Biggs et al. 1990). The original mutant allele, awd^{b3}, was identified as a mutation that caused late larval/early pupal lethality (reviewed in Timmons and Shearn 1997). Larvae that are heterozygous for the *awd* mutation have the same viability and developmental rate as wild-type controls, whereas larvae homozygous for the mutation stop growing and die in the middle of the third instar, immediately before pupation. Developmental defects in these third-instar–mutant larvae include morphologically abnormal and smaller wing discs and poorly differentiated ovaries and eye antennae and leg discs, which are larval tissues that normally develop into adult structures (Timmons and Shearn 1997). A null allele, awd^{KRs6}, caused similar but more severe developmental and morphological defects, demonstrating that *awd* is essential for development (Timmons and Shearn 1997). A third *awd* mutant, awd^{kpn} (referred to as "*killer of prune*"), is a proline-to-serine point mutation that induces no aberrant phenotype, even in the homozygous state. However, the mutation is lethal in the background of a mutation in a *prune* gene, indicating a functional interaction between the two genes and possibly a physical interaction between their products (Timmons and Shearn 1997).

Cellular differentiation is also strongly influenced by Nm23-H1 in mammals. Howlett et al. (1994) grew cells in a three-dimensional culture system, providing a basement membrane that mimics the environment of the mammary epithelium. This investigation showed that, when a metastatic breast cancer cell line with low endogenous Nm23-H1 expression was transfected with Nm23-H1, the cells recovered their normal morphology and patterns of growth (Howlett et al. 1994). Similarly, Nm23-H1 cDNA, when transfected into human pheochromocytoma cells, inhibited cellular proliferation and initiated neurite differentiation in the presence of nerve

growth factor (NGF), whereas downregulation of intracellular Nm23-H1 stimulated cellular proliferation and suppressed NGF-induced differentiation (Gervasi et al. 1996). Taken together, these genetic and cell-culture data provide strong evidence linking the differentiation process with Nm23-H1 protein levels.

Biochemical Activity of Nm23-H1/Mutational Analysis

Through what mechanism does Nm23-H1 control cellular differentiation and metastasis? Although there is no clear answer to this question, the structure and biochemical features of Nm23-H1 provide some clues. Nm23-H1 is a versatile kinase that can phosphorylate nucleoside diphosphate molecules and histidine residues on target proteins as well as autophosphorylate itself on at least two specific serine residues. Its best-studied function is as a nucleoside diphosphate kinase (NDPK): it transfers a terminal phosphate from a nucleoside triphosphate (NTP) to a nucleoside diphosphate, through a histidine-phosphate intermediate. The histidine phosphorylation occurs by autophosphorylation on histidine residue 118 (on the human sequence). Although NTP regeneration is required for both the energy needs of the cell and for signal transduction by G-proteins, this biochemical activity does not appear to explain either the metastatic suppressive nature of the protein or its role in development (reviewed in Steeg and Wagner 1997; also see below).

Just recently, we observed serine phosphorylation of Nm23-H1 in immunoprecipitates from mammary epithelial cell lysates (MacDonald et al. 1993). We detected phosphorylation of two serine-containing peptides and showed that these serine phosphorylations occurred downstream from, and were dependent on, the formation of a histidine-phosphorylation intermediate, indicating an autophosphorylation mechanism (MacDonald et al. 1993; Freije et al. 1997). The significance of the phosphate transfer from the histidine residue to the serine residue is still unknown, but this autophosphorylation possibly may regulate succeeding signaling events.

Site-directed mutagenesis experiments have allowed us to start to uncouple the different biochemical activities and biological functions. The Nm23-H1 mutants tested include (1) a proline 96–to-serine change (P96S), which is identical to the *Drosophila* mutation *awd*^{kpn}; (2) a serine 120–to-glycine substitution (S120G), which is identical to the mutation found in very aggressive neuroblastomas; and (3) a serine 44–to-glycine change (S44G), which removes a known phosphorylation site. Single clones of breast carcinoma cells transfected with mutant or wild-type Nm23-H1 cDNA were studied for in vitro motility activity. In a Boyden chamber assay, using serum or autotaxin as a stimulant, wild-type Nm23-H1 completely abrogated any cell movement. In contrast, the two mutants S120G and P96S reversed the wild-type effect, whereas S44G closely resembled that of the wild type (MacDonald et al. 1996). Thus, only two known sites of the Nm23-H1 protein, P96 and S120, were essential for its motility-suppression effect.

Purified, recombinant Nm23-H1 wild-type and mutant proteins showed no significant differences in NDPK activity or in serine and histidine autophosphorylation. In contrast, histidine protein-kinase activity exhibited a close correlation with the motility data: both P96S and S120G mutants were kinase deficient (Freije et al. 1997). Thus, the histidine kinase activity seems to act in a signaling pathway that directs the cells to remain static. These two mutations appear to alter the folding and assembly of Nm23-H1 complexes and to decrease the protein's stability in response to denaturation by heat and urea (Lascu et al. 1992, 1997; Chang et al. 1996). Whether these folding differences explain the decreased phosphotransfer of the proteins has yet to be determined, but they may influence the recognition of substrate molecules or the stability of protein complexes.

Signal Transduction/Two-Component Relay System

The major biochemical effect that seems to be related to motility behavior is the histidine protein-kinase activity of Nm23, an activity that is well studied in prokaryotes. In general, kinases of this type transfer a phosphate from the histidine-phosphate intermediate to a histidine or aspartate residue on an independent substrate. In response to some environmental stimuli, these proteins autophosphorylate on a histidine residue, initiating a series of phosphotransfer reactions. This relay system is referred to as a two-component signaling pathway. The autophosphorylated histidine protein kinase transfers the phosphate from its own histidine to an aspartate residue on a separate protein; this phosphate is subsequently passed on to a histidine of another protein and then to an aspartate residue of the next protein (reviewed in Appleby et al. 1996). In addition to prokaryotic systems, this ping-pong effect also has been detected in eukaryotic systems such as yeast and the cellular slime mold *Dictyostelium discoideum*. In yeast, activation of an analogous signal transduction pathway regulates activity of mitogen-activated protein kinases, which control both growth and mating responses (Maeda et al. 1994; Posas et al. 1996). Moreover, a bacterial homologue of Nm23-H1 has been reported to participate in a bacterial two-component system (Lu et al. 1996). Finally, the wild-type Nm23-H1 phosphorylates an aspartate residue on a 43-kD protein found in bovine brain-cell lysate, whereas the mutants P96S and S120G do not (Wagner et al. 1997). These results suggest that this phosphorelay system exists in mammalian cells

and may be important in the suppression of cell motility in premetastatic cells.

Summary

Nm23-H1 originally was discovered on the basis of its reduced RNA expression in very highly metastatic murine melanoma cell lines. Since that time, it has been shown to reduce the metastatic potential of highly aggressive tumors and to be an indicator of poor patient prognosis in many cancer types. Although LOH of *NME1* is prevalent in many tumors, this event does not predict reliably the overall levels of Nm23-H1 expression and therefore cannot be used as a genetic screen for the aggressive potential of a cancer. In addition, mutant forms of the gene exist; however, expression patterns seem to be the key to the differences observed between tumor cells of high and low metastatic potential. Differential Nm23-H1 expression may reflect transcriptional regulation and/or the stability of the Nm23-H1 transcript. The mechanism of action of the protein is still unknown; however, the protein does possess a unique kinase activity that is essential in other organisms. The histidine protein-kinase activity is the sole known activity that correlates tightly with the ability of Nm23-H1 to suppress cell motility. The identification of other components comprising this signaling pathway will be the next step toward unraveling the mysteries of this metastasis suppressor.

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