Suppressive Role of the Metastasis-related nm23-H1 Gene in Human Ovarian Carcinomas: Association of High Messenger RNA Expression with Lack of Lymph Node Metastasis

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Abstract

The nm23-H1 gene has been proposed as a metastasis suppressor gene. It is located on the long arm of chromosome 17, which is frequently deleted in ovarian cancer, and shows altered expression and structure in some advanced neoplasms. To evaluate the role of nm23-H1 in ovarian carcinogenesis, we have analyzed this gene in 66 primary human ovarian carcinomas at both the DNA and RNA levels. Despite the high frequency (76%) of nm23-H1 loss of heterozygosity (LOH), the complete absence of gene mutations in the coding portions of the retained allele clearly indicated that, in ovarian carcinomas, this gene does not function in the same way as do classic oncosuppressor genes. The relationship of clinicopathological parameters with nm23-H1 gene deletions and expression levels was also investigated. LOHs were more common in the serous and endometroid histotypes (85 and 93%, respectively), and the highest LOH frequency was detected in poorly differentiated tumors (89%). A significant relationship between nm23-H1 mRNA expression and lymph node metastasis was observed in high-grade tumors, which are intrinsically more invasive than are low-grade tumors. In particular, among the poorly differentiated tumors showing areas of undifferentiated solid carcinoma (classified as G1G2), lymph node-negative tumors displayed expression levels that were significantly higher than those of lymph node-positive tumors (P < 0.001). In conclusion, our data suggest that the nm23-H1 gene product may exert an inhibitory effect on the lymphatic dissemination of human ovarian tumors. However, several other factors, biological or time and patient dependent, influence the complex metastatic progression of ovarian tumors and may cooperate with nm23-H1 in the promotion or inhibition of this process.

Introduction

Ovarian carcinoma is the fourth commonest cancer in women and represents the most frequent cause of death from gynecological cancer. In the last few years, many biological studies have been addressed to the analysis of the genetic and molecular events occurring in ovarian tumors to identify the genes involved in their initiation and progression. In particular, studies on LOH have demonstrated a high incidence of chromosome 17q loss, indicating that one or more putative tumor suppressor genes, possibly implicated in ovarian carcinogenesis, might be located on this chromosomal arm. In familial breast and ovarian cancers, a linkage has been demonstrated with a hypothetical BRCA1 tumor suppressor gene on 17q12–q23 (1). However, detailed mapping studies (2, 3), together with the more recent characterization of the BRCA1 gene (4), seem to indicate that the deleted 17q portion in sporadic ovarian cancers is associated with a distinct tumor suppressor gene.

In ovarian neoplasias, LOH on 17q seems to be a relatively early event, which can occur before metastasis (2, 5). However, the comparison between tumors with localized disease and those with widespread disease showed that 17q allele loss increases as the stages become more advanced (6). Thus, loss of function of 17q-located gene(s) may be responsible for the frequent, rapid progression of ovarian adenocarcinomas to an advanced stage.

The search for the genes that may be involved in the promotion or suppression of the metastatic process has led to the identification of several interesting candidates. One of them, nm23-H1, is located on the long arm of chromosome 17 and has been proposed as a metastasis suppressor gene (7). Down-regulation of its expression during tumor progression has been reported in several in vitro and in vivo systems (7–12). Moreover, the evidence of nm23-H1 allelic deletions in several advanced human neoplasias (13, 14) and the detection of intragenic mutations in two cases of metastatic colorectal cancer (15) support a role for nm23-H1 in the inhibition of the metastatic process. However, the significance of nm23-H1 expression in human cancer seems to differ among the diverse tumor types, leading to contradictory conclusions regarding the biological properties of the nm23-H1 gene product (16–18).

The nm23-H1 and related nm23-H2 gene products correspond to the human nucleoside diphosphate kinase A and B subunits, respectively. Although the exact mechanism underlying the supposed nm23-related metastatic suppression remains unknown, several distinct intracellular functions of nm23/nucleoside diphosphate kinase could influence the metastatic process: microtubule assembly (19), signal transduction (20), transcription regulation (21), and cellular adhesion (22).

Considering the proposed function for the nm23 gene product, its altered expression or structure in various neoplasias, and its location within a commonly deleted region in ovarian cancers, we have performed a complete analysis of the nm23-H1 gene in a large series of primary ovarian tumor samples. The aim of this study was to evaluate the role of this gene in a type of neoplasm that usually manifests a very aggressive and invasive behavior and that can colonize multiple organs by different manners of diffusion.

Materials and Methods

Patients and Tissue Samples. This study considered 66 patients with common ovarian carcinoma who underwent surgery at the Department of Gynecological Oncology at our center. Only patients who had not been treated with surgery or chemotherapy and radiotherapy previously were included. Tumor samples were obtained immediately after surgery and were selected by a pathologist such that representative tissue sampling was routinely associated with sampling of adjacent tissue blocks for microscopic control examination. Stage of disease and grading were assigned according to Federation Internationale des Gynaecologistes et Obstetrises and TNM classifications, respectively. Among the 66 ovarian cancers, the following histological subtypes were distinguished: 37 serous, 16 endometrioid, and 4 mucinous adenocarcinomas; 6 undifferentiated carcinomas; 1 mixed serous and endometrioid adenocarcinoma; 1 mixed mesodermal Müllerian tumor; and 1 Brenner tumor. A total of
4 carcinomas were classified as well differentiated (G1), 12 as moderately differentiated (G2), 42 as poorly differentiated (G3), and 6 as undifferentiated (G4), whereas in 2 cases, the grading system was not applied. In the poorly differentiated (G3) tumor group, 19 cases showed areas of undifferentiated solid carcinoma (Fig. 1). These tumors were subclassified as G3/G4 and differed from the undifferentiated carcinomas (G4), which were entirely solid tumors or had very rare serous-like or endometrioid-like glands (Fig. 2). At the time of surgery, 5 patients were stage I, 6 were stage II, 35 were stage III, and 20 were stage IV. Finally, the patients with (N+) and without (N−) lymph node metastasis numbered 39 and 27, respectively. Specimens from multiple tumor sites (Ta, T1, and T2) were available for 18 patients. Peripheral blood lymphocytes or normal abdominal tissues of each individual were used as a source of normal DNA.

**LOH Analysis.** High molecular weight DNA was extracted from ovarian tumors and normal tissues with an automated nucleic acid extractor (Applied Biosystems). RFLP and microsatellite analyses were performed to search for allelic deletions of the nm23-H1 gene. In the first experimental approach, BglII- and EcoRI-restricted DNAs were analyzed by Southern blotting, as described previously (23). Southern blots were hybridized to a random primer 32P-labeled 900-bp BamHI fragment of the pNM23-H1 plasmid (24). Microsatellite analysis was carried out by amplification of the polymorphic CA-repeat region of the nm23-H1 gene (25). PCRs were performed in 10 µl of reaction buffer containing 1.5 mM MgCl2, 0.1 µg DNA, 10 pmol of each primer, 2.5 µM of each deoxyribonucleoside triphosphate, 0.1 µM [α-32P]dCTP (10 µCi/µl; Amersham, Buckinghamshire, United Kingdom), and 0.5 units Taq polymerase (Promega, Madison, WI). Templates were denatured for 4 min at 94°C in a thermal cycler (MJ-PT100), and then subjected to 20 cycles of PCR with incubations of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. After amplification, 2 µl of the 32P-labeled PCR products were mixed 1:1 with 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol; heat denatured; and electrophoresed on 6% denaturing polyacrylamide gel.

The hybridized Southern membranes and the vacuum-dried polyacrylamide gels were exposed to X-ray films at —20°C, and the intensity of the autoradiographic signal for 1 allele in the tumor DNA was interpreted as LOH.

**SSCP Procedures.** The oligonucleotide primers for SSCP analysis of the five nm23-H1 exons were synthesized according to the method of Baffico et al. (26). All of the tumor DNAs were amplified by PCR as described above, with the exception of [α-3P]dATP (10 µCi/µl) (Amersham), which was used instead of [α-32P]dCTP. Annealing temperatures varied from 50°C to 60°C. After amplification, one-tenth of the reaction was mixed 1:1 with 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol; heat denatured; and electrophoresed on 6% denaturing polyacrylamide gel.

The hybridized Southern membranes and the vacuum-dried polyacrylamide gels were exposed to X-ray films at —80°C, and the intensity of the autoradiographic signals was determined by densitometric scanning (Hoefer, New- castle, United Kingdom). Loss of or significant reduction in (at least 50%) the autoradiographic signal for 1 allele in the tumor DNA was interpreted as LOH.

**RESULTS**

LOH of the nm23-H1 gene was evaluated in Southern blot experiments by the BglII and EcoRI RFLPs, which identify allelic bands of 7.6 or 2.3 kb and 4.6 or 2.4 plus 2.2 kb, respectively (Ref. 26; Fig. 3). These data were integrated and confirmed by analysis of the polymorphic CA-repeat region of the same gene (Ref. 25; Fig. 3). Of the 66 patients, only 8 (12%) were not informative by this multiple approach. Of the remaining 58 cases, 44 (76%) exhibited the deletion of one nm23-H1 allele at the tumor level. When specimens from multiple tumor locations were available for a single patient (18 cases), the same allelic pattern was observed. Amplification of the nm23-H1 gene was never detected. In BglII-digested KOV83Ta DNA, a minor constant band above 21 kb was absent (Fig. 3), but digestion with six other restriction enzymes invariably produced a normal pattern (data not shown).

The possibility that nm23-H1 function could be affected by mutations in the coding or splicing regions of the gene was investigated using the SSCP technique. The five fragments examined appeared to be normal in all of the tumor DNAs, suggesting the absence of sequence abnormalities. Exon 1 SSCP was able to detect clearly a common polymorphism (Ref. 26; Fig. 4), and imbalance in the intensity of the electrophoresed bands was generally in accordance with the data on LOH obtained with the above-mentioned analyses.

**nm23-H1 expression** was studied by Northern blot in 52 ovarian carcinomas, 10 of which were at more than one tumor site (Fig. 5). All ovarian tumor samples expressed the 0.8-kb transcripts, but 46 of 52...
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Fig. 3. Representative nm23-H1 LOH analyses in ovarian tumors. Top, Southern hybridization to BglII-digested (left) and EcoRI-digested (right) DNA samples from normal (N) and tumor (Ta) tissues. The 900-bp nm23-H1 probe identified allelic bands at 7.6 and 2.3 kb (with BglII-digested DNA) or at 4.6 and 2.4 plus 2.2 kb (with EcoRI). Bottom, electrophoretic separation of nm23-H1-amplified fragments containing a CA-repeat sequence. Homozygous (a), heterozygous (b), and deleted (c and d) tumors are shown. Arrowheads, the allele lost in tumor DNAs. Arrowhead with asterisk, the anomaly in tumor KOV83T.

Fig. 4. Exon 1 SSCP analysis of tumor DNAs. In this case, the different patterns are generated by a polymorphic site: the 1A and 1B bands correspond to allele 1, whereas the mobility shifts of 2A and 2B bands are caused by the polymorphism of allele 2. In the plate, tumor DNA samples homozygous for allele 1 (KOV31Ta) or allele 2 (KOV31Ta, KOV14Ta, KOV25Ta, KOV36Ta, and KOV44Ta), both heterozygous (KOV44Ta) and with partial or total deletion of one allele (the others), are shown.

Fig. 5. Representative example of nm23-H1 mRNA expression analysis. After hybridization with a 168-bp nm23-H1-specific probe (top), a glyceraldehyde-3-phosphate dehydrogenase probe was used for control of RNA loading (bottom). Three cases with multiple tumor sites (Ta and Tb) are shown.

Fig. 6. Relationship between nm23-H1 mRNA level, grading, and lymph node metastasis in ovarian carcinoma patients. The mRNA a.u. values of lymph node-negative (N−) and lymph node-positive (N+) tumors are plotted to the left and to the right of the vertical axes, respectively. A horizontal arrow separates low-expressing (<10 a.u.) from high-expressing (>10 a.u.) tumors. ■, serous; □, endometrioid; ▲, undifferentiated; ◆, mucinous; ○, mixed.

tumors exhibited low expression levels (range, 0.7–10 a.u.), and in only six tumors was a decidedly higher nm23-H1 mRNA content observed (15.5–42.2 a.u.; Fig. 6). Interestingly, the expression level maintained the same magnitude order in all of the tumor sites of a patient, independent of its primary or metastatic origin (Fig. 5).

Regarding clinicopathological parameters (Table 1), LOH was highly frequent in the serous (28 of 33) and endometrioid (13 of 14) tumors but was rare or absent in the undifferentiated (1 of 5) and mucinous (0 of 3) carcinomas (χ² overall = 23.38; P < 0.001).

The incidence of LOH was similar in the groups of lymph node-negative (18 of 25) and lymph node-positive (26 of 33) patients. Moreover, among early-stage tumors, two of four (stage I) and three of five (stage II) showed nm23-H1 allelic deletion, but this anomaly was more frequent in advanced-stage tumors: 25 of 31 tumors in stage III and 14 of 18 in stage IV.

Finally, none of the three well-differentiated tumors and only one of five undifferentiated tumors showed allelic loss at nm23-H1, in contrast with the moderately or poorly differentiated tumors, which exhibited a high incidence of LOH (8 of 11 and 34 of 38, respectively). When the different histotypes were considered separately (Table 1), a significant increase in the LOH frequency (0, 67, 93, and 100%), with increasing G values, was apparent in the serous tumor group (χ² trend = 11.67; P < 0.001). An upward trend of LOH frequency (50, 50, 89, and 90%) with stage progression was also observed, but in this case, the statistical significance was not reached.
The relationship between clinicopathological data and nm23-H1 mRNA expression was also examined (Table 2). Mean values of a.u. were considered for the tumors studied at multiple sites. There was no considerable variability in expression among the various histotypes and between tumors with distant metastases at the time of surgery (stage IV: mean, 5.47 ± 4.75) and tumors without them (stages I-III: mean, 6.71 ± 8.11). Taking into account the single histotypes, the difference between expression levels of serous tumors in stage IV (mean, 3.70 ± 2.12) and stages I-III (mean, 7.53 ± 9.75) was more pronounced compared with the whole series, but still not statistically significant. Lower expression levels were observed in tumors with nodal dissemination (mean, 5.75 ± 7.17) as compared to lymph node-negative tumors (mean, 7.23 ± 7.52), but this slight down-regulation had no statistical significance. However, only 1 of 31 N+ tumors (3%) expressed more than 10 a.u. of nm23-H1 mRNA, whereas a greater percentage of N− tumors (5 of 21 = 24%) was in the high-expressing group (Fig. 6). The two-sided Fisher’s exact test showed a significant difference in distribution (P < 0.05).

Concerning grading, a clear upward trend or downward trend of a.u. values with grade progression was not evident. However, the highest nm23-H1 mRNA expression levels were displayed by G3/G4 tumors (mean, 10.90 ± 6.85). In particular, the expression of G3/G4 serous (mean, 9.56 ± 6.48) and endometrioid (mean, 11.4 ± 7.26) tumors was greater than that of the more differentiated G1/G2, and G3 tumors (means, 5.22 ± 8.80 for serous and 3.46 ± 1.13 for endometrioid tumors). The Mann-Whitney U test attributed statistical significance to these differences (P < 0.05). nm23-H1 mRNA expression levels were also evaluated in tumors of different grade in relation to lymph node involvement (Fig. 6). A significant difference was observed between G1/G2 N− (mean, 20.75 ± 2.98) and G3/G4 N+ (mean, 6.96 ± 2.11) tumors (Z = 2.758; P < 0.001). Four of the five N− tumors expressing more than 10 a.u. were G3/G4; therefore, high nm23-H1 expression seems to be restricted mainly to the high-grade tumors without lymph node metastasis.

Analysis of the other clinicopathological data did not show remarkable differences in the distribution of nm23-H1 LOH and expression levels.

### Discussion

LOH at loci on 17q is a very common event in invasive epithelial ovarian tumors, with frequencies ranging from 40 to 77% (2, 3, 5, 6, 29, 30). It is likely that this chromosomal arm contains one or more oncosuppressor genes responsible for these tumors. However, in the majority of cases, the deletions encompass the entire chromosomal arm, making it difficult to define the hot spot(s) and to identify the gene(s) possibly involved. In fact, at present, rather confused data exist concerning the number and the localization of 17q target sequences implicated in ovarian tumorigenesis. On the other hand, it is possible that the tendency to extensive rather than localized subchromosomal losses reflects the presence of many cooperating genes.

The nm23-H1 gene on 17q21 is certainly one of the most interesting candidates. In fact, the findings of allelic and homozygous deletions, mutations, and down-regulation of nm23-H1 in human cancer demonstrate that this gene shares a mechanism of altered regulation with known suppressor genes (14, 15, 31). Moreover, several studies correlating the nm23-H1 status with some clinicopathological data indicate a role for this gene in metastatic tumor progression rather than in its initiation (9–11, 13). In accordance with this, transfection of the cDNA in human tumor cells resulted in reduced metastatic potential in vivo and reduced mobility and colonization ability in vitro (32–34).

To establish the role of this supposed metastasis suppressor gene in ovarian carcinogenesis and the significance of structural and expression abnormalities, we have performed an extensive analysis of the gene at both the DNA and RNA levels in a large number of ovarian carcinomas.

On the basis of 58 informative tumors, a very high percentage of nm23-H1 LOH (76%) was found. This finding was superimposable to that reported in other papers by the combined analysis of several polymorphic loci on 17q (2, 29, 30). This high frequency could suggest that the nm23-H1 locus represents or is very close to a 17q hot spot. However, in this study, the complete absence of gene mutations
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in the coding portion of the retained nm23-H1 allele supports clearly the notion that other adjacent genes with oncogenic potential (erbB2, RARA, phb, 17 hsd2, and TIMP2) are more likely to be involved in ovarian carcinogenesis. Therefore, the loss of an nm23-H1 allele might not be the most relevant selective event but could result from a wide chromosomal loss, which includes several genes.

KOV83T, was the only tumor DNA displaying, by Southern blotting, a structural anomaly other than LOH. However, the data available on the restriction sites of the nm23-H1 gene indicate that the missing large BgIII fragment does not correspond to this gene. It could represent a related gene, such as nm23-H2, or, more likely, a pseudogene (35).

nm23-H1 LOH was considered by histological subtype. Allelic deletion was more frequently associated with the serous and endometrioid tumors (85 and 93%, respectively). In accordance with previous papers evidencing a lower incidence of 17q LOH in mucinous tumors (2, 29), we detected the presence of both nm23-H1 alleles in three mucinous informative cases. Despite the low number of cases, the absence of LOH seemed to be related to this particular histological subtype rather than to a concomitant low grade, as suggested by Foulkes et al. (29). In fact, only one mucinous tumor was G3; the other two were G4. In contrast with other authors (2, 29), we found a low incidence of LOH in undifferentiated carcinomas (20%). This discrepancy could be explained by the different diagnostic criteria used by pathologists for this histotype (36).

Although there were no statistically significant differences, LOH was more common in late-stage carcinomas, but nm23-H1 losses were seen in all stages. Moreover, the same allele was lost in the primary and metastatic sites of tumors, confirming that 17q LOH may be an early event in the progression to metastatic ovarian cancer (2, 6).

Similar considerations may be made from the observation that LOH frequency increased as the histological grade increased (from 0% in G1 to 100% in G4/G5 serous tumors). In the progression to a higher grade, tumors often become increasingly malignant and invade and metastasize more widely and rapidly. This progression to anaplasia may be favored by 17q LOH. However, only 20% of nm23-H1 LOHs were found in G4 tumors, which were associated with the undifferentiated histotype. The G4 group could represent a distinct category and, apparently, is not involved in the malignant progression that implies loss of cell differentiation.

Our study indicates that the differences in mRNA expression levels between ovarian tumors with hemogenous or distant metastasis (stage IV) and those of the other clinical stages and between lymph node-positive and lymph node-negative tumors are not statistically significant. Both of these observations are in contrast with the data of Mandai et al. (37), which demonstrated a statistically significant inverse association between nm23-H1 expression level and lymph node and/or distant dissemination in ovarian carcinomas. Similar relationships have also been described for other tumors, such as breast, gastric, and colorectal cancer (10, 12, 31). The wide dispersion of expression level values in our study might partially explain this discrepancy. In addition, the mechanism of ovarian tumor invasion may be very complex, involving several biological or time- and patient-dependent factors that could drastically affect the metastatic diffusion process and partially mask the nm23-H1 effects. However, the nm23-H1 mRNA expression levels in G4/G5 tumors without lymph node metastasis were significantly higher than those in G4/G5 lymph node-positive tumors, suggesting a role for nm23-H1 expression in the control of the metastatic process. It may be that a high nm23-H1 mRNA expression exerts an inhibitory effect on the lymphatic dissemination, and this inhibition may become ineffective when nm23-H1 drops below a threshold level and other favorable conditions are in place. The effect of an elevated nm23-H1 expression level seems to play a role in preventing lymphatic dissemination mainly in high-grade tumors (G4/G5), which are intrinsically more invasive.

Significantly higher nm23-H1 mRNA expression levels were observed in G2/G3 tumors, particularly in the serous or endometrioid subgroups. Therefore, in ovarian carcinomas, as in squamous cell lung carcinomas and prostate cancer (18, 38), higher nm23-H1 expression seemed to be associated with the more malignant, less differentiated tumors. However, this association is not in contrast with the proposed suppressive function of the nm23-H1 gene on tumor progression. In fact, a more accurate analysis, which took into account distribution of expression levels in respect to grading in combination with other clinicopathological parameters, clearly evidenced the above-mentioned prevalence of high expression levels in the subgroup of G4/G5 tumors without lymph node involvement. The lower mean expression level shown by G4 tumors compared with G2/G3 tumors can be justified by the lack of lymph node-negative G4 cases in this series.

In conclusion, the analysis of a large number of human ovarian tumors has evidenced a high frequency of nm23-H1 LOH but the complete absence of point mutations or other structural anomalies in the retained allele. Therefore, the nm23-H1 gene is not likely to be involved as a classical oncosuppressor gene in ovarian carcinogenesis. A tendency toward higher nm23-H1 expression associated with lack of lymph node metastasis was observed. However, the majority of both lymph node-negative and lymph node-positive tumors displayed low expression levels, indicating that the metastatic progression of ovarian carcinomas depends on the balance between the promoting and inhibiting effects exerted by several metastasis-related factors (17). Among these, nm23-H1 could represent a “weak” element that is unable, by itself, to affect drastically the lymphatic and hematogenous diffusion of ovarian carcinomas.

REFERENCES


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