Expression of Metastasis-related nm23-H1 and nm23-H2 Genes in Ovarian Carcinomas: Correlation with Clinicopathology, EGFR, c-erbB-2, and c-erbB-3 Genes, and Sex Steroid Receptor Expression

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ABSTRACT

To verify the role of metastasis-related nm23 genes in carcinogenesis and progression of ovarian carcinoma, we analyzed the mRNA levels of the nm23 genes of both isoforms, H1 and H2, together with those of the epidermal growth factor receptor, the c-erbB-2, and the c-erbB-3 genes in 45 ovarian carcinomas and 5 benign cystadenomas. Expressions of nm23 gene products/nucleoside diphosphate kinases, epidermal growth factor receptor, erbB-2 protein, and sex steroid receptor status in ovarian carcinomas were also examined by immunohistochemistry. The mRNA levels of nm23-H1 and nm23-H2 were higher in carcinoma tissues compared with benign tumors (H1, P < 0.01). The mRNA levels of c-erbB-2 and c-erbB-3 were also elevated in carcinoma tissues, and there was a positive correlation between mRNA levels of the nm23-H1 and the c-erbB-2 genes (r = 0.58; P < 0.05). Correlation of immunohistochemical staining between nucleoside diphosphate kinases and erbB-2 protein was also observed in ovarian carcinoma tissues. Sex steroid receptor positivity was related to a higher expression of nucleoside diphosphate kinases. Expression levels of the nm23 genes in ovarian carcinomas were not related to either histological subtype or local extension and peritoneal dissemination. Among stage III ovarian carcinomas, however, tumors possessing lymph node metastasis showed significantly lower nm23-H1 mRNA levels than those without nodal involvement (P < 0.05). Stage IV carcinomas also exhibited lower nm23-H1 and nm23-H2 expression levels compared with other stages (P < 0.05). These results suggest that expression of the nm23 genes, especially nm23-H1, is activated, accompanied by c-erbB-2 and c-erbB-3 overexpressions, in early stages of the carcinogenic process of ovarian carcinoma and reduction of nm23-H1 expression occurs in association with lymph nodal and/or distant metastasis.

INTRODUCTION

Cancer metastasis is a complex process involving a sequential series of multistep genetic events (1, 2) and a result of imbalance between stimulatory and inhibitory genes for metastasis (3, 4). The nm23 gene was initially cloned as a metastasis suppressor gene (5), whose expression leads to reduction of tumor metastasis without affecting tumor growth (6). Reduced nm23 expression has been shown to be associated with the presence of metastasis and/or a patient's poorer prognosis in breast carcinoma (7–10), hepatocellular carcinoma (11), malignant melanoma (12), and gastric carcinoma (13). However, high nm23 expression has been reported to correlate with carcinogenesis or progression in colon carcinoma (14) and neuroblastoma (15). Therefore, the significance of nm23 expression in human cancers may be different according to the organs in which the tumors develop. Little is known about nm23 gene expression in ovarian cancer with respect to its carcinogenesis and tumor progression.

Recent studies have demonstrated several genetic alterations in ovarian cancer including inactivation of tumor suppressor genes such as p53 (16, 17) and activation of oncoproteins such as K-ras (18–20), c-myc (21, 22), c-fms (23), epidermal growth factor receptor (EGFR)3 (24), and c-erbB-2. The c-erbB-2 gene is a well-recognized protooncogene encoding a Mr 185,000 transmembrane protein, which is homologous to EGFR and has tyrosine kinase activity (25). It has been shown that amplification and/or overexpression of c-erbB-2 is associated with poorer prognosis of ovarian cancer patients (26–28). The third EGFR family protooncogene, c-erbB-3, has recently been identified (29, 30), and the significance of its expression in ovarian cancer is still to be clarified.

Accordingly, we investigated nm23 mRNA levels in benign and malignant epithelial tumors of the ovary. It is now known that there are at least two isoforms of the nm23 gene which are designated as nm23-H1 and nm23-H2 in the human (31) and that the expression level of nm23-H1 is much lower than that of nm23-H2 in most human tissues. Products encoded by the nm23-H1 and the nm23-H2 genes correspond to NDPK A and B, respectively (32). RT-PCR has made it possible to quantify various kinds of gene expression in a small amount of total RNA simultaneously (33) and also to discriminate expressions of homologous but distinctively different genes such as the nm23-H1 and the nm23-H2 genes by choosing appropriate primers for PCR. In the present study, we carried out RT-PCR to study gene expressions of nm23-H1, EGFR, c-erbB-2, and c-erbB-3 and Northern blot hybridization to analyze nm23-H2 expression. ER and PR status is another prognostic parameter in ovarian cancer, and its positivity is usually related to good prognosis (34). Thus, protein level expressions of NDPK, EGFR, erb-B2, ER, and PR were also examined by immunohistochemistry.

MATERIALS AND METHODS

Tissue Samples. Fresh surgical specimens of ovarian epithelial tumors were obtained from 50 patients who underwent bilateral salpingo-oophorectomy and hysterectomy. They consisted of 5 benign cystadenomas, 4 carcinomas of LMP, and 41 frankly invasive carcinomas (Table 1). Clinical staging was performed according to the classification of the International Federation of Obstetrics and Gynecology (35). Stage III carcinomas consisted of 6 cases with lymph node metastasis and 15 cases without lymph node metastasis. In four cases of stage III, samples could be obtained both from primary tumors and peritoneal metastatic lesions. Normal ovaries were obtained from six patients who underwent surgery for benign gynecological diseases with written forms of consent and were used as a control. The materials obtained immediately after surgical procedure were snap frozen in liquid nitrogen and stored at −80°C. The samples for RNA analysis were prepared carefully under a dissecting microscope to eliminate inappropriate components. Samples for immunohistochemistry were also obtained from the same tissues adjacent to the parts from which RNA was extracted.

Probes and Primers. Pairs of oligonucleotide primers for PCR were designed to insert an intron in the corresponding genomic sequence to eliminate amplification from genomic DNA. Their sequences, the length of products, and annealing temperature are summarized in Table 2.

3 The abbreviations used are: EGFR, epidermal growth factor receptor; NDPK, nucleoside diphosphate kinase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; ER, estrogen receptor; PR, progesterone receptor; LMP, low malignant potential; β2-MG, β2-microglobulin.

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RESULTS

mRNA Detection by Northern Blot Hybridization and RTPCR. Expressions of the nm23-H2 gene were detected in all the cases examined (Fig. 1A), whereas nm23-H1 transcript was not detectable in any case by Northern blot analysis. On the other hand, the mRNA expressions of nm23-H1, EGF R, c-erbB-2, and c-erbB-3 were detected by RT-PCR in all the cases (Fig. 1B). To determine the number of PCR cycles appropriate for quantification, PCR was performed from 20 to 50 cycles at an increase of 5 cycles. The ratio of PCR products of target genes to those of the β2-MG gene was reasonably constant between 25 and 45 PCR cycles (data not shown). Therefore, in the subsequent experiments the values at 30 PCR cycles were defined as the expression of target genes.

nm23 mRNA Levels in Ovarian Tumors. Table 1 shows the mRNA levels of the nm23-H1 and the nm23-H2 genes in primary tumors. The expression levels of the nm23-H1 gene were significantly higher in carcinoma tissues compared with those in benign tumors (P < 0.01) and in normal ovaries (P < 0.01; Table 1; Fig. 2). The expression levels of the nm23-H2 gene were also higher in carcinomas than in benign tumors and in normal ovaries, although the differences were not significant. In LMP cases, nm23-H1 expression was between benign tumors and frank carcinomas.

Table 1 mRNA expression of the nm23 gene in relation to clinical stage and histological subtype

<table>
<thead>
<tr>
<th>nm23-H1</th>
<th>nm23-H2</th>
</tr>
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<tbody>
<tr>
<td>Normal ovary</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>Benign tumor</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>LMP</td>
<td>0.68 ± 0.26</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0.90 ± 0.35</td>
</tr>
<tr>
<td>Stage I</td>
<td>0.92 ± 0.31</td>
</tr>
<tr>
<td>Stage II</td>
<td>0.98 ± 0.25</td>
</tr>
<tr>
<td>Stage III</td>
<td>0.95 ± 0.38</td>
</tr>
<tr>
<td>Stage IV</td>
<td>0.62 ± 0.33</td>
</tr>
<tr>
<td>Serous</td>
<td>0.90 ± 0.36</td>
</tr>
<tr>
<td>Mucinous</td>
<td>0.45 ± 0.32</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>1.01 ± 0.30</td>
</tr>
<tr>
<td>Clear</td>
<td>0.83 ± 0.24</td>
</tr>
</tbody>
</table>

* Total case number.  
* Cases in which nm23-H2 expression was determined.  
* Consisted of two serous, two mucinous, and one endometrioid.  
* Significantly higher expression (P < 0.01) compared with benign tumor and normal ovary.  
* Significantly lower expression (P < 0.05) compared with 1-III stages.

Table 2 Sequences of amplification primers shown in 5′ → 3′ orientation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF R</td>
<td>U CAAACCTGCTGAGGCGGGA</td>
<td>175</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>T DGGTCATAGGGCTGAT</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td>U AATGTCGGCAAGATTCCGG</td>
<td>178</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>G DCTGCCCAGCACTCTCATT</td>
<td>169</td>
<td>31</td>
</tr>
<tr>
<td>c-erbB-3</td>
<td>U TGAATTACGCGTCTACATACA</td>
<td>120</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>D ATCTTCAAAAAGATTGA</td>
<td></td>
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</tr>
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</table>

* Size of the amplified fragments obtained with each pair of primers.  
* Referred articles to design the primers.

Immunohistochemistry. Rabbit polyclonal antibody against the rat NDPK was provided by Dr. Naomichi Kimura, Department of Molecular Biology, Metropolitan Institute of Gerontology, Tokyo, Japan. This antibody is known to react with both nm23-H1 and nm23-H2 proteins and to be suitable for immunohistochemical analysis of human samples (38). Mouse monoclonal antibodies against the external domain of EGFR (Ab-1) and against the external domain of erbB-2 protein were purchased from Oncogene Science (New York, NY) and Triton Diagnostics (Chicago, IL), respectively. Immunohistochemistry was performed on cryostat sections using a Histogen Rabbit System (Biomed, Foster, CA) for NDPK and a Monoclonal Detector Kit (Biomeda) for EGFR and erbB-2 proteins. Immunohistochemistry for ER and PR was performed on serial cryostat sections using ER-ICA and PgR-ICA monoclonal kits (Abbott, North Chicago, IL).

Statistical Analysis. Differences of mRNA levels between two independent groups were evaluated by Mann-Whitney's U test, and the results of immunohistochemistry were analyzed by χ² test. The Spearman rank correlation coefficient was used to evaluate the correlation between the paired values.

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With respect to clinical stage of ovarian carcinomas, both nm23-H1 and nm23-H2 showed almost similar levels of expression in stages I, II, and III, respectively (Table 1). Interestingly, stage IV carcinomas exhibited lower levels of expression of nm23-H1 and nm23-H2 compared with stage I-III carcinomas (P < 0.05, respectively). In four cases of stage III carcinomas, the expressions of nm23-H1 and nm23-H2 could be compared between the primary tumors and their corresponding peritoneal metastatic lesions. The expressions of nm23-H1 and nm23-H2 were lower in the metastatic lesions in three of four cases, although the difference were not significant. (Fig. 3). Among stage III carcinomas, nm23-H1 mRNA levels of primary tumors were significantly lower in cases with lymph node metastasis than in those negative for lymph node metastasis (P < 0.05; Fig. 2). On the other hand, nm23-H2 mRNA levels were not different, irrespective of lymph node involvement.

With regard to the histological subtype of ovarian carcinoma, expressions of the nm23-H1 and the nm23-H2 genes were lower in mucinous tumors compared with other histological subtypes. However, the difference was not statistically significant (Table 1). Neither nm23-H1 nor nm23-H2 mRNA level showed significant correlation with the grade of histological differentiation of carcinomas (data not shown).

EGFR, c-erbB-2, c-erbB-3 mRNA Levels in Ovarian Tumors. Fig. 4 shows expression levels of EGFR, c-erbB-2, and c-erbB-3 in the primary tumors. The expression levels of the EGFR gene showed no significant difference among all the ovarian tumors and normal ovaries examined. In contrast, the expressions of the c-erbB-2 and the c-erbB-3 genes were significantly higher in frank carcinomas compared with benign tumors (P < 0.05) and with normal ovaries (P < 0.01). Expressions of the c-erbB-2 and the c-erbB-3 genes in LMP were also significantly higher than those in normal ovaries (P < 0.05). With regard to histological subtypes, mucinous carcinomas showed higher EGFR expression compared with carcinomas of other types (data not shown).

Correlations of nm23-H1 expression with nm23-H2, EGFR, c-erbB-2, and c-erbB-3 expressions are shown in Fig. 5. Among these,
nm23-H1 expression showed moderately positive correlation with c-erbB-2 expression ($r = 0.58; P < 0.01$). However, the c-erbB-2 gene did not show significant reduction of mRNA levels as observed in nm23 expressions in association with distant metastasis.

**Immunohistochemical Detection of nm23 Product/NDPK, EGFR, and erbB-2 Proteins.** Immunohistochemical localizations of NDPK, EGFR, c-erbB-2, and c-erbB-3. Moderately positive correlations ($r = 0.58; P < 0.01$) were found between nm23-H1 and c-erbB-2 expressions. ☀️ normal ovary; ☼️ benign tumor; ☯️ LMP and carcinoma.

**DISCUSSION**

The present study demonstrated the expression levels of metastasis-related nm23 genes of both nm23-H1 and nm23-H2 isoforms in ovarian epithelial carcinomas, benign tumors, and normal ovaries. The mRNA levels of nm23-H1 were significantly higher in frank carcinomas than in benign ovarian tumors. The expression levels of the nm23-H2 gene in ovarian carcinomas were also higher than in benign tumors, although the difference was not significant. This suggests that activation of nm23 gene expression, especially that of nm23-H1, occurs during tumorigenesis of ovarian carcinoma, and this is consistent with the previous report regarding nm23 expression in colon carcinoma (14). In malignant melanoma (12) and gastric carcinoma (13), it is also reported that nm23 expression is higher in tumors compared with corresponding normal tissues. LMP is a unique category of ovarian carcinoma intermediate between clearly benign and frankly malignant tumors (39). In the present study, nm23-H1 expression levels of LMP ranged between those of benign tumors and frank carcinomas, suggesting that nm23 gene expression is well correlated with the histopathological features of epithelial ovarian tumors.

Clinical stage is the most important prognostic factor for ovarian carcinoma (39), reflecting the pathways of expansion: direct spread by local extension (stage II); intraperitoneal dissemination (stage III); and regional lymph node metastasis (stage IIIc) or hematogenous distant metastasis (stage IV). Our study showed that the expression levels of both the nm23-H1 and the nm23-H2 genes were not significantly different between stage I carcinomas and primary lesions of more advanced stage II and III carcinomas. Neither nm23-H1 nor nm23-H2 mRNA level was significantly different between primary tumors and peritoneal disseminated lesions in all of the four matched pairs in stage III carcinomas. These findings suggest that nm23-H1 and nm23-H2 expressions are not related to either direct extension or seeding into the peritoneal cavity of the carcinoma cells. Interestingly, however, among stage III tumors, nm23-H1 expression in primary lesions of the carcinomas with lymph node metastasis was significantly lower than that of tumors without nodal metastasis. On the other hand, expression of the nm23-H2 gene was not significantly different between these node-positive and negative tumors. Expression levels of the nm23-H1 and the nm23-H2 genes of stage IV carcinomas were lower compared with those of other stages ($P < 0.05$). Thus, it appears that the expression level of the nm23-H1 gene in the primary tumor is inversely associated with lymph node metastasis and/or distant metastasis and that reduction of nm23 expression levels may be a late event during the progression of ovarian carcinomas.

To date, there are only a limited number of reports on nm23 expression in which the two isoforms of the nm23 genes are separately measured, presumably due to their high homology in nucleotide sequences and/or due to low expression of the nm23-H1 gene. It has been reported that the expressions of the two genes may be regulated independently (31). In the present study the expression of nm23-H1 rather than nm23-H2 appeared more closely related to tumorigenesis and progression of ovarian carcinomas.

Immunohistochemical study using polyclonal antibody against NDPK revealed the intracytoplasmic localization of the nm23 gene products (NDPKs) both in normal and neoplastic cells of the ovary. The staining intensity for NDPKs in ovarian carcinoma cells was stronger than that in the surface epithelial cells of normal ovaries from which ovarian carcinoma is thought to arise. However, we need further study to determine correlation between transcriptional and translational levels of the nm23 genes using antisense-specific antibodies against human NDPKs because the antibody used in this study was raised against rat NDPK and reacts with both isoforms of nm23-H1 and nm23-H2 products by immunoblotting (38).

NDPKs have been reported to be associated with a variety of GTP-binding proteins and to regulate the function of G-proteins in signal transduction (40). This prompted us to study the possible

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**Table 3 indicates the relationship between ER/PR status and NDPK immunoreactivity.** All of the eight ER- and PR-positive carcinomas showed moderate to strong staining for NDPK. On the other hand, in 20 ER- and PR-negative carcinomas, NDPK expression was negative in 5 (25.0%) cases and positive in 15 (75.0%) cases.
Fig. 6. Immunohistochemical localization of NDPK, erbB-2 protein, and PR in a case of endometrioid adenocarcinoma of the ovary. A, malignant tumor cells showing intracellular localization for NDPK. B, staining of erbB-2 protein observed on the cell membrane. C, PR positivity observed in the nucleus of the tumor cells. D, negative control of the same sample. Normal rabbit serum was used instead of anti-NDPK antibody. A-D, × 200.

Table 3 Relationship between NDPK positivity and ER/PR status in carcinomas

<table>
<thead>
<tr>
<th>ER and/or PR</th>
<th>NDPK</th>
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<tbody>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>5 (25)</td>
</tr>
<tr>
<td>+ ± +</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
</tr>
</tbody>
</table>

Relationship between nm23 mRNA levels and expression levels of other signal transduction-related oncogenes such as EGFR family members. Overexpression of the c-erb-B2 gene in ovarian carcinoma has been well documented in association with poor prognosis (26, 28). A previous study using immunohistochemistry has also shown higher expression of erbB-2 protein in ovarian carcinomas than in normal surface epithelium of the ovary and benign ovarian cystadenomas (41). In agreement with these reports, the current study revealed significantly higher mRNA levels of the c-erbB-2 gene in carcinoma tissues than in benign tumors. The significance of EGFR in association with the behavior of ovarian carcinoma is controversial, i.e., EGFR positivity reportedly indicates positive (42) and inverse (43) correlation with a better patient’s prognosis. To the best of our knowledge, systematic analysis of EGFR gene expression in ovarian carcinoma has not been described. Expression of erbB-3 protein is histochemically observed in the germ cells of the fetal ovary and granulosa cells of the adult ovary (44), but its expression in ovarian carcinoma has not yet been reported. The current study revealed that the c-erbB-3 gene expression was elevated in ovarian carcinomas compared with benign tumors and was closely related to c-erbB-2 expression. Therefore, activation of both the c-erbB-2 and the c-erbB-3 genes may contribute to carcinogenesis of ovarian carcinoma.

It is reported that ovarian and breast cancer cell lines transfected with c-erbB-2 exhibited reduced nm23 RNA level (45). In this study, however, there was a significant positive correlation between the mRNA levels of the nm23-H1 and the c-erbB-2 genes in ovarian carcinoma. This correlation was also observed by immunohistochemical staining of the serial sections for nm23 products/NDPKs and erbB-2 protein. Further studies are needed to clarify the functional interaction between the nm23 genes and the c-erbB-2 gene in ovarian carcinoma.

In the present study, immunohistochemical expression of NDPK was positive in all of the ER/PR positive carcinomas, whereas 33% of the cases of ER/PR negative tumors were negative for NDPK. This is consistent with the study that breast carcinomas with high nm23 expression exhibit ER positivity (46). Expressions of both the nm23-H1 and the nm23-H2 genes are also reportedly higher in estrogen-dependent MCF-7 cells compared with the estrogen-independent MDAMB-435 cell line (31).

In summary, fluctuation of metastasis-related nm23-H1 gene expression may play an important role in progression and metastasis of ovarian carcinoma. Although the intracellular function of nm23 gene
ACKNOWLEDGMENTS

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