Nuclear receptor DAX-1 in human common epithelial ovarian carcinoma: An independent prognostic factor of clinical outcome

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DAX-1 is a member of the nuclear receptor superfamily and is thought to be involved in the regulation of steroidogenesis. Its expression has been detected primarily in endocrine neoplasms such as adrenocortical as well as pituitary tumors in human, but its biological roles have not been examined well in sex steroid-dependent neoplasms. The aim of this study is to detect the expression of DAX-1 in common epithelial ovarian carcinomas in order to evaluate its possible biological significance. DAX-1 immunoreactivity was examined using immunohistochemistry. The correlation between the status of DAX-1 immunoreactivity and clinicopathological parameters and disease-free survival of the patients in a series of 60 cases of common epithelial ovarian carcinoma was examined. The status of DAX-1 immunoreactivity was evaluated using H score. DAX-1 immunoreactivity was widely detected in the nuclei of common epithelial ovarian carcinoma cells. There was a significant positive correlation between DAX-1 immunoreactivity and clinical staging (P=0.0241), tumor grade (P=0.0115), the residual size of the tumor (P=0.0014) and Ki-67 labeling index (P=<0.0001). In univariate survival analysis, a significant association was detected between DAX-1 immunoreactivity and shortened patient survival (P=0.0157). Other significant prognostic parameters were clinical stage, residual size of tumor and Ki-67. In multivariate analysis, DAX-1 immunoreactivity, clinical stage, residual size of tumor and Ki-67 all turned out to be independent prognostic factors for shortened survival. In conclusion, DAX-1 immunoreactivity is considered to be a new independent marker of poor prognosis or adverse clinical outcome in patients with epithelial ovarian carcinoma, possibly through altering *in situ* steroids production. (Cancer Sci 2003; 94: 980–985)

Common epithelial ovarian carcinomas are characterized by a broad spectrum of biological behavior ranging from tumors associated with excellent prognosis and response to therapy to those with poor prognosis and a high mortality rate. This marked variety is related to the fact that many patients are diagnosed at an advanced clinical stage.

A number of prognostic factors have been proposed for common epithelial ovarian carcinoma, and are expected to be useful in deciding which patients need additional postoperative treatment and whether they may get benefit from treatment in terms of prolongation of disease-free and overall survival. The most important prognostic factors are clinical staging, residual size of the tumor, tumor grade and the patient's age.[1, 2] In addition to these relatively well established parameters, other prognostic parameters that have been proposed include estrogen and progesterone receptors (ER and PR), Ki-67 labeling index (LI),[3–5] and the preoperative serum marker CA125.[6] The identification of new prognostic parameters should contribute to further improvement of treatment and the clinical outcome of the patients.

DAX-1 (Dosage-sensitive sex reversal Adrenal hypoplasia congenita critical region on the X chromosome, gene 1) is a member of the nuclear receptor superfamily.[7] Its expression is largely restricted to steroidogenic tissues such as adrenal cortex, ovary, Leydig cells and other endocrine cells such as testicular Sertoli cells, pituitary gonadotropes, ventromedial hypothalamic nucleus cells and others.[8, 9] DAX-1 acts as a negative regulator of steroid production by repressing the expression of steroidogenic acute regulatory protein (StAR),[10–12] which is essential for the first and rate-limiting step in steroid biosynthesis and the transfer of cholesterol to the inner mitochondrial membrane,[10, 13, 14] where side-chain cleavage P450 (P450scc) converts cholesterol into pregnenolone. Pregnenolone is further transformed into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD).[15] In addition, DAX-1 has also been demonstrated to negatively regulate the transcription activity of steroidogenic factor-1 (SF-1),[16] which is another member of the nuclear receptor superfamily that positively regulates the expression of multiple cytochrome P450 steroid hydroxylases and StAR.[17]

Among human neoplasms, the status of DAX-1 has been extensively examined in adrenal gland and adrenocortical tumors,[16, 18] but its roles in sex steroid-dependent neoplasms, in which *in situ* steroid production and metabolism are considered to play important roles, have not been examined well. In our present study, we examined the status of DAX-1 in common epithelial ovarian carcinoma and correlated the findings with prognostic factors, such as tumor stage, the size of the residual tumor, tumor grade, the age of the patients, the preoperative serum marker CA125 and the LI of Ki-67, ER and PR, in order to evaluate its possible clinical and biological significance in patients with common epithelial ovarian carcinoma.

Materials and Methods

Patients' population. The study materials were 4 normal ovaries and 60 primary epithelial ovarian carcinomas, which were divided as follows: 33 cases of serous carcinoma, 11 cases of endometrioid carcinoma and 16 cases of clear cell carcinoma. All these archival specimens were retrieved from surgical pathology files at Tohoku University Hospital, Sendai. Patients' ages ranged from 36 to 74 years (median 50 years). Clinicopathological characteristics of these carcinoma cases are summarized in Table 1. Tumor stage was assessed according to the International Federation of Gynecology and Obstetrics. Tumors were graded according to the histological grading system proposed by Silverberg.[19] Histological types were determined according to the criteria of the World Health Organization (WHO). Both grading and histological subtypes were evaluated by three different observers (MA, TM, JA). Preoperative serum CA125 levels were obtained from the review of medical records of epithelial ovarian carcinoma patients included in this

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study. CA125 was determined by immunoradiometric assay (Centocor, Malvern, PA). The research protocol was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai.

Primary antibodies. Rabbit polyclonal antibody for DAX-1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies for PR (PR-AB, PR-A: hPRa7 and PR-B: hPRa2) were purchased from NeoMarkers (Fremont, CA). ERα and Ki-67 monoclonal antibodies were purchased from Immunotech. (Marseilles, France). The characteristics of the primary antibodies used in immunohistochemistry are summarized in Table 2.

Immunohistochemistry. All specimens were fixed for 24 h in 10% formalin and embedded in paraffin wax. Sections of 3 μm were cut and placed on clean glass slides (Matsunami, Tokyo). After deparaffinization, the slides for DAX-1, PR-AB, PR-A, PR-B and Ki-67 were placed in citric acid buffer (2 mmol/liter citric acid and 9 mmol/liter trisodium citrate dehydrate, pH 6.0) and autoclaved for 5 min at 121°C and those for ERα were placed in boiling citric acid buffer and heated in microwave oven for 15 min. The slides were subsequently allowed to cool at room temperature, and washed in 0.01 *M* phosphate-buffered saline (PBS). The slides were then reacted with 1% normal rabbit or goat serum for 30 min at room temperature. The dilutions of primary antibodies used in our study were as follows: DAX-1, 1/500; Ki-67, 1/100; PR-AB, 1/50; PR-A, 1/100; PR-B, 1/100 and ERα, 1/50. They were incubated with tissue sections at 4°C for 18 h.

The sections were washed in PBS, and the endogenous peroxidase activity was blocked by placing the slides in 100% methanol with 0.3% hydrogen peroxide for 30 min at room temperature (22°C). The slides were then incubated with biotinylated anti-mouse or anti-rabbit immunoglobulin for 30 min at room temperature, and subsequently incubated with peroxidase-conjugated streptavidin for 30 min at room temperature, using the Histofine Kit (Nichirei, Tokyo). The sections were washed with 0.01 *M* PBS, and the antigen-antibody complexes were visualized by immersion in DAB solution (1 mmol/liter 3,3′-diaminobenzidine tetrahydrochloride (DAB), 50 mmol/liter Tris-HCl buffer, pH 7.6 and 0.006% hydrogen peroxide). The sections were counterstained with hematoxylin. Human tissue sections used as positive controls in this study were as follows: adrenal gland for DAX-1, endometrium for PR; PR-A, PR-B and breast cancer for ERα. As a negative control, normal rabbit or mouse IgG was used instead of primary antibodies. No specific immunoreactivity was detected in these sections.

Scoring of immunoreactivity. Semiquantitative analysis of immunoreactivity of DAX-1 (so-called H score) was performed in this study as reported by McCarty *et al*.[20] with some modifications.[21] Briefly, more than 500 tumor cells were counted in each case, and the H score was generated by adding together 2×% for strongly stained nuclei, 1×% for weakly stained nuclei and 0×% for negative or scattered cells representing less than 10%, giving a possible range of 0–200.[21–23]

Ki-67, ERα and PR immunoreactivity were scored in more than 500 tumor cells for each case, and the percentage of immunoreactivity regardless of immunointensity, i.e. LI, was obtained.

Statistical analysis. Statistical analysis was accomplished using SAS software (StatView, version 5.0.1, Cary, NC). The significance of prognostic factors was assessed using both univariate and multivariate analysis. The statistical significance of the correlation between expression of DAX-1 and clinicopathological parameters was performed using regression analysis. For survival analysis, the Kaplan-Meier method and log-rank test were used to generate and to compare the different survival curves. Multivariate progression analysis using the Cox proportional hazards model was performed to test the independent value of each parameter in predicting overall survival of the patients. *P* values <0.05 were considered significant.

Table 1. Clinicopathological parameters of patients with common epithelial ovarian carcinoma

Total number	60
Patients age (years)[1]	50.9±1.2
Histology	
Serous	33 (55%)
Endometriod	11 (18.3%)
Clear cell	16 (26.7%)
FIGO stage	
I	14 (23.3%)
II	24 (40%)
III	6 (10%)
IV	16 (26.7%)
Silverberg grade	
G1	19 (31.7%)
G2	18 (30%)
G3	23 (38.3%)
Residual size of tumor (mm)[1]	1.8±1.97

1) Values represent mean±SD.
Other values represent number of cases and percentages.

Table 2. Characteristics of primary antibodies used in immunohistochemistry

Antibodies	Source	Optimal dilution
DAX-1 (polyclonal)	Cruz Biotechnology, Inc. (Santa Cruz, CA).	1/500
PR-AB (monoclonal)	NeoMarkers (Fremont, CA)	1/50
PR-A: hPRa7 (monoclonal)	NeoMarkers (Fremont, CA)	1/100
PR-B: hPRa2 (monoclonal)	NeoMarkers (Fremont, CA)	1/100
ERα (monoclonal)	Immunotech. (Marseilles, France)	1/50
Ki-67: MIB-1 (monoclonal)	Immunotech. (Marseilles, France)	1/100

Results

Immunohistochemistry of DAX-1 in common epithelial ovarian carcinomas. DAX-1 immunoreactivity was detected in the nuclei of epithelial cells and stromal cells in 4 cases of normal human ovary (100% of cases) (Fig. 1-A), 30 cases of serous carcinoma (91% of cases) (Fig. 1-B), 11 cases of endometrioid carcinoma (100% of cases) (Fig. 1-C) and 15 cases of clear cell carcinoma of the ovary (94% of cases) (Fig. 1-D). The H score value (mean±SD) for DAX-1 was 156.957±8.05 PR-(A+B), PR-A, PR-B, ERα and Ki-67 LI (mean±SD) values were 41.52%±28.4%, 8%±11.3%, 40%±27.4%, 19.3%±21% and 49.4%±15.7%, respectively.

Correlation between DAX-1 immunoreactivity and clinicopathological parameters. The correlations between DAX-1 immunoreactivity and clinicopathological parameters of the cases were first examined by univariate analysis. There was a significant positive correlation between DAX-1 immunoreactivity and FIGO stage (P=0.0241), tumor grade (P=0.0115), the residual size of the tumor (P=0.0014) and Ki-67 LI (P<0.0001), as shown in Fig. 2 and Table 3. However there was no significant correlation between DAX-1 immunoreactivity and age, preoperative CA125, PR-(A, B, A+B) or ERα of the patients.

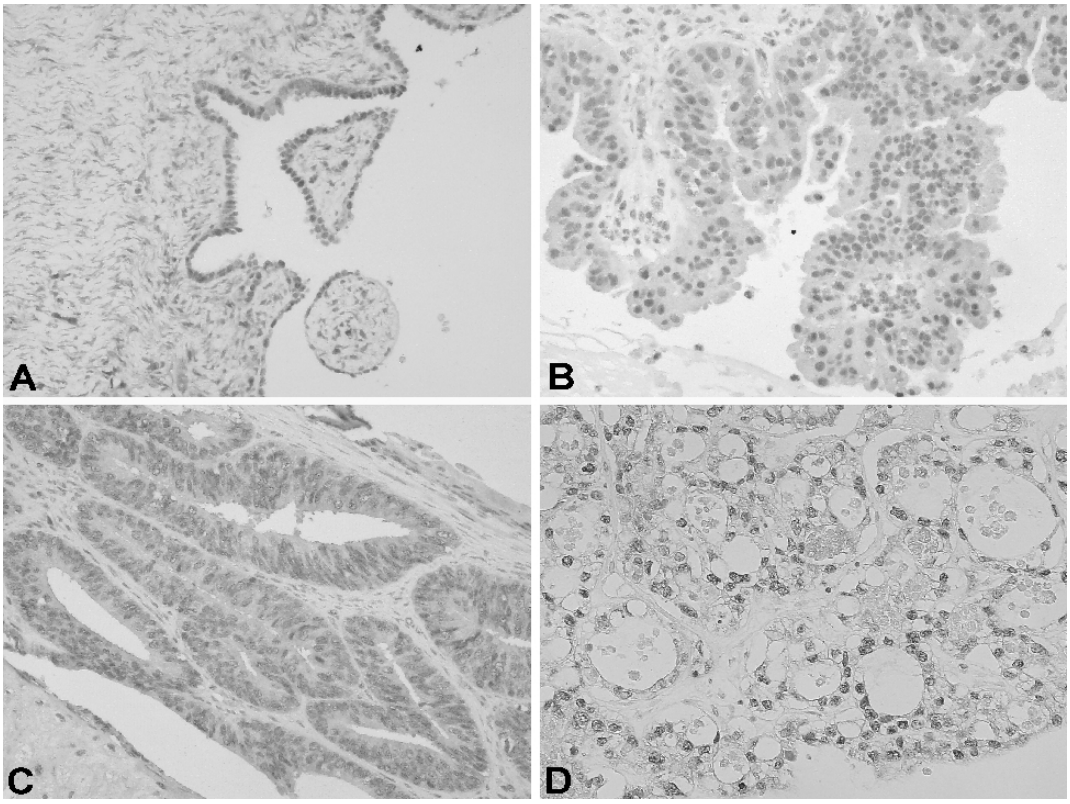

Fig. 1. DAX-1 immunohistochemistry in tissue specimens of normal human ovary and epithelial ovarian carcinoma. DAX-1 immunoreactivity was detected in the nuclei of epithelial and stromal cells of normal human ovary and epithelial ovarian carcinoma. A: DAX-1 immunoreactivity in normal surface epithelium of human ovarian tissue. B: DAX-1 immunoreactivity in serous type of epithelial ovarian carcinoma. C: DAX-1 immunoreactivity in endometrioid type of epithelial ovarian carcinoma. D: DAX-1 immunoreactivity in clear cell type of epithelial ovarian carcinoma. (original magnification ×200)

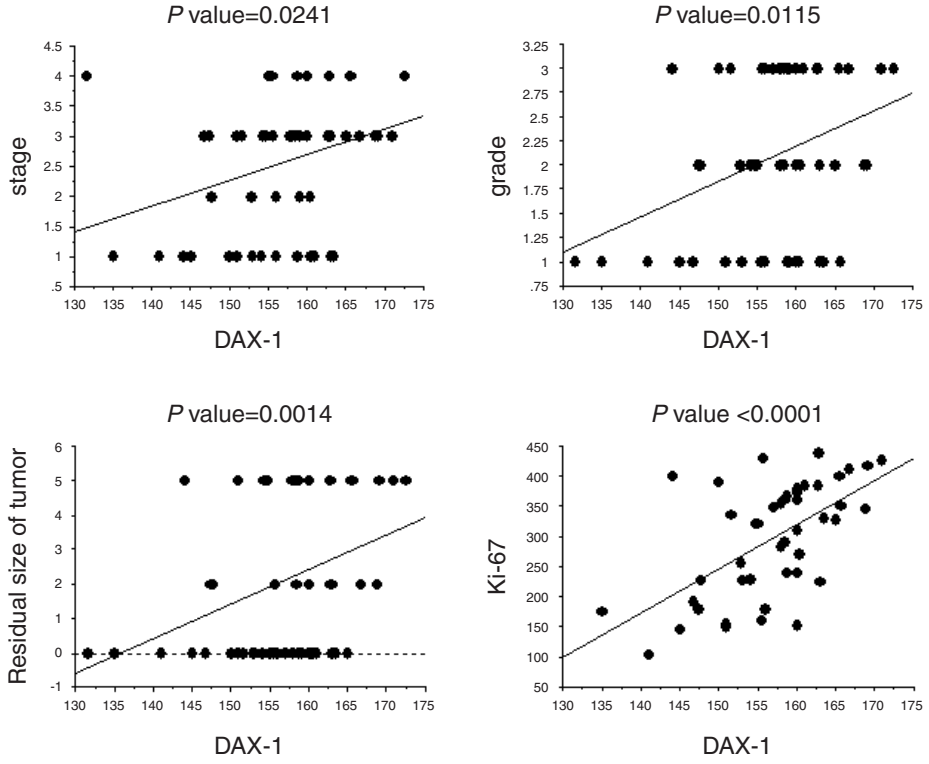

Fig. 2. Bivariate scattergram showing correlation between H score for DAX-1 and other clinicopathological factors. Upper left: Correlation between DAX-1 H score and FIGO stage (P=0.0241). Upper right: Correlation between DAX-1 H score and tumor grade (P=0.0115). Lower left: Correlation between DAX-1 H score and residual size of the tumor (P=0.0014). Lower right: Correlation between DAX-1 H score and Ki-67 LI (P<0.0001).

DAX-1 immunoreactivity and patient survival. Among 60 patients examined, 35 died of ovarian carcinoma, and 25 were alive. The significance of DAX-1, age, tumor grade, FIGO stage, residual size of tumor, preoperative serum marker CA125, proliferation marker Ki-67 and differentiation markers PR and ER in relation to patients' survival was evaluated by univariate and multivariate analyses.

When DAX-1 was included with the other covariates (age, tumor stage, tumor grade, residual size of the tumor, preoperative CA125, Ki-67, ER and PR) of the patients in the univariate analysis, significant inverse correlations were detected between DAX-1 immunoreactivity (P=0.0157), residual size of the tumor (P<0.0001), tumor stage (P=0.0143) and Ki-67 LI (P=0.0261) and the clinical outcome of the patients. Cumulative survival curves were calculated according to the Kaplan-Meier method. The log-rank test was used to compare survival curves (Fig. 3, Table 4).

Multivariate survival analysis. The independent prognostic value of DAX-1 immunoractivity, as well as other clinicopathological parameters that were found to be significant in univariate analysis, i.e. residual size of tumor, clinical stage and Ki-67 immunoreactivity, was further evaluated by multivariate progression analysis based on the COX proportional hazard model. DAX-1 immunoreactivity (P=0.031), residual size of the tumor (P=0.0014), FIGO stage (P=0.0373) and Ki-67 immunoreactivity (P=0.0316) turned out to be independent prognostic factors as evaluated by both univariate and multivariate statistical analyses (Table 4).

Discussion

Information on DAX-1 expression has been broadly restricted to steroidogenic tissues such as human adrenal gland,[8, 9, 16, 18, 22, 23] since the *DAX-1* gene encodes an orphan nuclear hormone receptor essential for normal fetal development of the adrenal cortex.[19] In addition, the mutations in the *DAX-1* gene are well known to be responsible for congenital X-linked adrenal hypoplasia associated with hypogonadotropic hypogonadism.[7]

In this study, we examined the immunohistochemical localization of DAX-1 in normal human surface epithelium, as well as different carcinomas arising from surface epithelial cells. DAX-1 immunoreactive protein was localized in the nuclei of normal surface epithelium of human ovary, as well as in the stromal cells of all normal ovary specimens examined. The results in our study are in good agreement with those of a recent study by Sato *et al*.[22] They demonstrated the expression pat-

Table 3. Relationship between DAX-1 expression and various clinicopathological factors in total 60 patients with common epithelial ovarian carcinoma

	DAX-1 immunoreactivity	
	P value	*r*
Patients age (years)	0.7616	0.042
FIGO stage (I, II, III, IV)	0.0241	0.307
Sliverberg grade	0.0115	0.335
Residual size of tumor (mm)	0.0014	0.416
Preoperative CA125	0.0706	0.261
PR H score	0.2842	0.151
ER H score	0.9921	0.001
Ki-67 LI	<0.0001	0.592

Table 4. Univariate and multivariate analysis of overall survival

Variable	Univariate	Multivariate
	P	P
Patients age (years)	0.2509	
FIGO stage (I, II, III, IV)	0.0143	0.0373
Sliverberg grade	0.417	
Residual size of tumor (mm)	<0.0001	0.0014
Preoperative CA125	0.3469	
PR H score	0.7824	
ER H score	0.6485	
Ki-67 LI	0.0261	0.0316
DAX-1 H score	0.0157	0.031

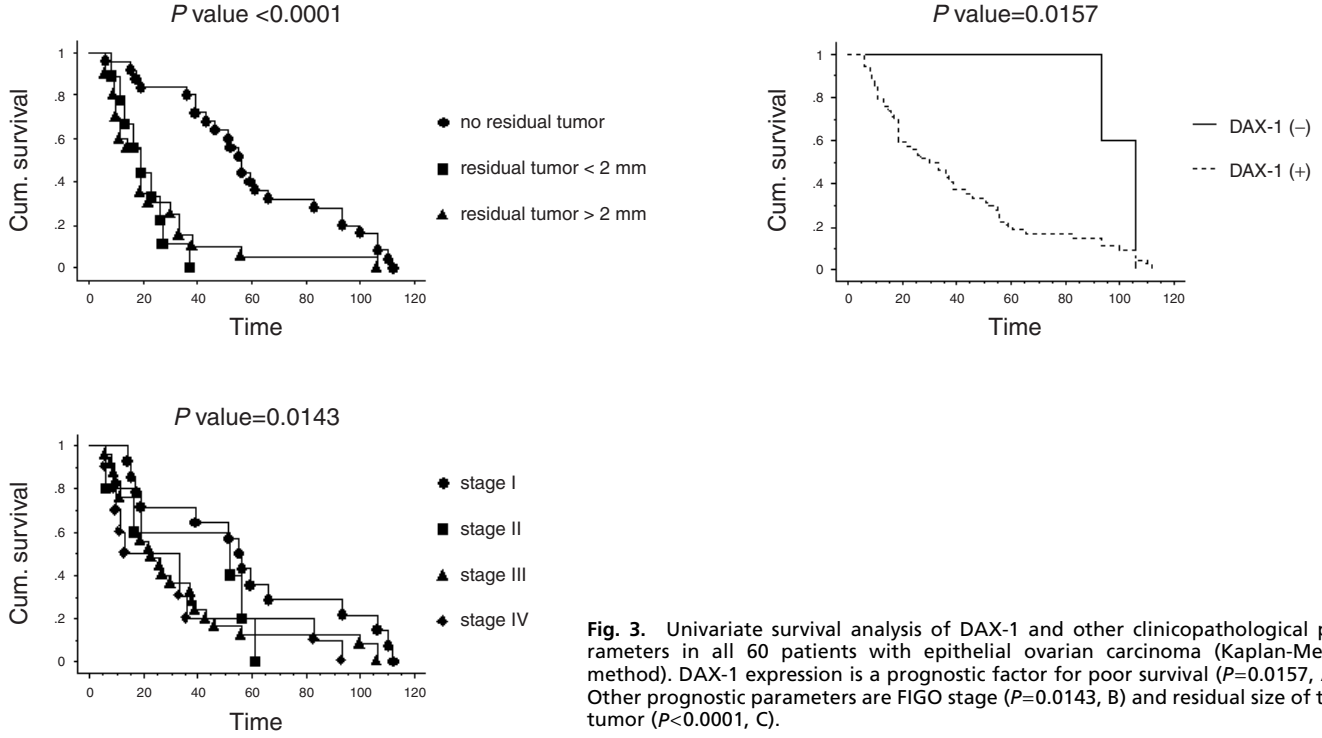

Fig. 3. Univariate survival analysis of DAX-1 and other clinicopathological parameters in all 60 patients with epithelial ovarian carcinoma (Kaplan-Meier method). DAX-1 expression is a prognostic factor for poor survival (P=0.0157, A). Other prognostic parameters are FIGO stage (P=0.0143, B) and residual size of the tumor (P<0.0001, C).

terns of DAX-1 in different cell types and follicular stages in normal cycling human ovaries, in which DAX-1 immunoreactivity was predominantly detected in granulose cells in ovarian follicle and corpora lutea. In addition, DAX-1 immunoreactivity was relatively high in follicular stages from primordial to non-dominant rather than in preantral and dominant follicular stages. Based on the above results, the authors concluded that DAX-1 may play a role in the modulation of Ad4BP (Adrenal 4 Binding Protein)/SF-1-dependent transcription of steroidogenic enzymes involved in human ovarian steroidogenesis.

Among neoplasms, DAX-1 expression was detected primarily in adrenocortical neoplasm and pituitary adenoma.[16, 18, 24, 25] Many previous studies on DAX-1 expression in adrenocortical and pituitary neoplasms have demonstrated the presence of DAX-1 in non-functioning adrenal and pituitary adenomas. DAX-1 expression was, however, low or even absent in aldosterone-producing adenomas, adrenocortical carcinomas of the adrenal gland as well as in GH-secreting and PRL-secreting adenomas of the pituitary gland. These results indicate that DAX-1 is one of the factors regulating steroid biosynthesis in these neoplasms.

DAX-1 expression has not been well studied in sex steroid-dependent neoplasms. This is the first study to examine the status of DAX-1 in malignant epithelial ovarian carcinoma in order to evaluate the prognostic impact of this hormonal receptor on these carcinomas. The prognostic impact of DAX-1 was evaluated on the basis of detection of its expression in epithelial ovarian carcinoma in a total of 60 patients, and the results were correlated with other putative prognostic factors of ovarian tumors and with the overall survival of the patients.

In the present study, DAX-1 immunoreactivity was detected in the nuclei of epithelial cells of ovarian carcinoma and was directly correlated with tumor grading, residual size of the tumor, FIGO stage and Ki-67 labeling index. DAX-1, advanced clinical stage, increasing size of the residual tumor and higher values of Ki-67 LI all turned out to be independent factors for poor prognosis in patients with common epithelial ovarian carcinoma. It is then important to elucidate the possible mechanisms of DAX-1 expression in carcinoma cells in relation to aggressive biological behaviors commonly seen in epithelial ovarian carcinoma patients.

Previous studies have demonstrated the importance of DAX-1 in human steroidogenesis and in the regulation of steroid hormone production, as described above. Lalli *et al*.,[10] measured the production of pregnenolone and progesterone in Y-1/hDAX-1 cells (DAX-1-expressing Y-1 cells derived from adrenocortical tumors). They reported that the production of both pregnenolone and progesterone is extremely low in Y-1/hDAX-1 cells and that in these cells, the first steps of the steroidogenic cascade are almost completely suppressed. They further demonstrated that DAX-1 inhibits steroid production and metabolism of Y-1 cells at multiple levels.

Progesterone has been demonstrated to be involved in ovarian cancer development and progression.[26] In addition, recent studies have demonstrated *in situ* production of progesterone in human common epithelial ovarian carcinoma.[27] Progesterone has also been considered to directly mediate decreased tumor growth[28] through inhibition of cell proliferation and induction of apoptosis.[29, 30] The above findings suggest that DAX-1 may inhibit progesterone hormone production in the tumor tissue through repressing multiple biochemical steps in the steroidogenic cascade. This decreased level of progesterone production may result in increased cellular proliferation and decreased apoptosis of carcinoma cells. The highly significant statistical correlation between DAX-1 immunoreactivity and Ki-67 LI in this study is also consistent with this hypothesis. However, further investigations are required.

This study is the first to demonstrate the significant prognostic value of DAX-1 expression in any of the human tumors. In addition, the analysis of DAX-1 in tumor cells of common epithelial ovarian carcinoma may be helpful in the management of the patients, possibly representing a surrogate marker of hormonal or endocrine therapy. In summary, DAX-1 immunoreactivity was detected in the nuclei of epithelial ovarian carcinoma cells, and positive DAX-1 immunoreactivity was shown to be an independent marker for poor prognosis in common epithelial ovarian carcinoma.

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