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Original Article

Clinical Relevance of TAp73 and ΔNp73 Protein Expression in Ovarian Cancer: A Series of 83 Cases and Review of the Literature

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Summary: The p73 gene gives rise to the full-length transactivation competent TAp73 and the N-terminally truncated isoform ΔNp73, which inhibits TAp73 and wild-type p53. The clinical relevance of TAp73 and ΔNp73 protein expression has not yet been evaluated in ovarian cancer. TAp73 and ΔNp73 expression was examined using immunohistochemistry and reverse transcription–polymerase chain reaction in 83 and 64 ovarian cancer specimens, respectively. A yeast-based assay and subsequent sequencing were performed to analyze the *p53* mutational status. TAp73 and ΔNp73 protein expression was found in 73 of 83 (88%) and 48 of 83 (57.8%) ovarian cancer samples, respectively. The majority of cases showed immunostaining in both the nucleus and cytoplasm of tumor cells. TAp73 and ΔNp73 protein expression correlated with messenger RNA quantification in 25 of 64 (39.1%) and 37 of 64 (57.8%) cancer specimens, respectively. TAp73 and ΔNp73 protein expression was not associated with the *p53* mutational status, clinicopathologic parameters, and prognosis of the examined ovarian cancer cases. Although TAp73 and ΔNp73 protein expression did not possess prognostic significance for ovarian cancer in this study, a potential clinical role of p73 isoforms cannot be definitively excluded due to limitations of immunohistochemistry.
Key Words: p73—ovarian cancer—immunohistochemistry.

The p53 family member p73 possesses a complex genomic organization and gives rise to multiple p73 isoforms. Alternative splicing of the P1 promoter transcript generates both full-length *TAp73* and *ΔN'p73*, whereas an alternative P2 promoter in intron 3 produces *ΔNp73*. Importantly, *ΔN'p73* and *ΔNp73* transcripts encode the same protein product. As this N-terminally truncated p73 protein ΔNp73 lacks the transactivation domain, it acts as a powerful dominant-negative inhibitor of both wild-type p53 and TAp73 either by direct competition for DNA-binding sites or by the formation of heterocomplexes. In addition, ΔNp73 is able to induce phosphorylation of the retinoblastoma tumor suppressor, causing its inactivation. ΔNp73 also possesses p53-independent functions and has been reported to affect the expression of various genes (1). ΔNp73 protein expression

From the Departments of Gynecology and Obstetrics (G.H., A.B., M.C., E.M-H., D.R., C.M., A.G.Z, N.C.); Medical Statistics, Informatics, and Health Economics (H.U.), Medical University Innsbruck, Innsbruck; Second Department of Surgery (H.U.), University of Occupational and Environmental Health, Kitakyushu, Japan; Department of Obstetrics and Gynecology (R.Z.), Molecular Oncology Group, Medical University of Vienna; and Ludwig Boltzmann Gesellschaft (R.Z.), Cluster Translational Oncology, Vienna, Austria.

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has been significantly associated with impaired survival in patients with gastric and hepatocellular cancer (2,3). In addition, ΔNp73 protein expression has been an independent prognostic marker in lung and cervical cancer (4,5). In ovarian cancer, we previously reported that ΔNp73 messenger RNA (mRNA) expression plays a role in the response to platinum-based chemotherapy and prognosis (6).The aim of this study was to evaluate the clinical relevance of TAp73 and ΔNp73 protein expression in primary ovarian cancer.

MATERIALS AND METHODS

Patients

Eighty-three patients, who underwent primary surgery for ovarian cancer in the Department of Gynecology and Obstetrics, Medical University Innsbruck, were recruited. Clinicopathologic parameters (Table 1) and follow-up data were obtained by retrospective chart review. With the exception of 8 patients presenting with FIGO stage I cancer, all patients received subsequent platinum-based chemotherapy. This study was approved by the ethics committee (project number UN3507) of the institution.

Immunohistochemistry

Three-micrometer-thick sections were obtained from each of the 83 formalin-fixed, paraffin-embedded samples of primary lesions. All specimens were stained with hematoxylin and eosin for histopathologic diagnosis. After deparaffinization, sections were placed in Epitope Retrieval Solution [DakoCytomation, Glostrup, Denmark 10 mmol citrate buffer (pH 6.0), part of HercepTest] in a preheated water bath (98°C, 40 minutes). Endogenous peroxidase was blocked by ChemMate Peroxidase Blocking Solution (DakoCytomation) for 5 minutes. The primary antibodies TAp73 (H-79; Santa Cruz Biotechnology, CA) and ΔNp73 (4) diluted 1:400 in ChemMate Antibody Diluent (DakoCytomation), were applied for 30 minutes, followed by the secondary antibody (ChemMate Dako Envision; DakoCytomation) for 30 minutes and 3,3′-diaminobenzidine (DakoCytomation ChemMate Dako Envision) for 10 minutes. After counterstaining with hematoxylin (diluted 1:2 in demineralized water for 5 minutes; DakoCytomation), sections were dehydrated and mounted. All incubation steps were performed at room temperature. Two variants of negative controls were done with antibody diluent omitting primary antibody and Negative Control Reagent (part of HercepTest) instead of the specific primary antibody.

All slides were evaluated in a blind manner. Cells were classified as positive for TAp73 and ΔNp73 when the cytoplasm or the nucleus or both were stained in more than 10% of tumor cells. For further statistical analyses, TAp73 and ΔNp73 expression scores were divided into 2 groups: positive and negative.

TABLE 1. *Clinicopathologic characteristics of the examined primary ovarian cancer cases*

Characteristics	*n*
Age at diagnosis (yr), median age (range)	60 (24–88)
FIGO stage	
I	16
II	4
III	48
IV	15
Histologic subtype	
Serous	34
Endometrioid	15
Mucinous	28
Others	6
Grading	
II	48
III	35
Residual disease	
No residual disease	34
Residual disease <2 cm	21
Residual disease ≥ 2 cm	28

Reverse Transcription–Polymerase Chain Reaction

p73 isoform-specific reverse transcription–polymerase chain reaction (RT-PCR) was available in 64 ovarian cancer specimens from our previous study (6). Transcript levels were arbitrarily divided at the 50th percentile into high and low expression groups.

Yeast-based Functional Assay for p53 and Sequence Analysis

To detect inactivating *p53* mutations in the ovarian cancer specimens, the functional yeast-based assay was used as described previously (6,7). In brief, functional yeast-based assay is based on the transcriptional activity of wild-type versus mutant *p53* alleles in a yeast reporter system. *p53* mRNA was reverse transcribed, amplified by PCR, and cotransformed into *Saccharomyces cerevisiae* with a linearized yeast homologous recombination expression vector carrying the 5′ and 3′ ends of the *p53* open reading frame. Wild-type p53, which activates transcription of the yeast *ADE2* gene that encodes the

phosphoribosyl-aminoimidazole carboxylase, results in white colonies, whereas mutant alleles lack transcriptional activity and result in smaller, red colonies. DNA from at least 2 red colonies was sequenced to characterize the *p53* mutations.

Statistical Analyses

The Statistical Package for the Social Sciences for Windows 18.0 software (SPSS, Inc., Chicago, IL) was used for all analyses. The χ^2 or the Fisher's exact test was applied for the pairwise comparison of TAp73 and ΔNp73 protein expression, *p73* isoform transcript levels, *p53* mutational status, and clinicopathologic parameters. Survival probabilities were calculated by the product limit method of Kaplan and Meier. Differences between groups were tested using the log-rank test. The Cox proportional hazards model was used for multivariate analysis to assess the independence of different prognostic factors. P values <0.05 were considered statistically significant.

RESULTS

Positive TAp73 immunostaining was detected in 73 of 83 (88%) ovarian cancer specimens (Fig. 1). Of these, 59 (80.8%) specimens showed both nuclear and cytoplasmatic TAp73 localization, whereas staining was confined to the nucleus of tumor cells in 9 (12.3%) cases and to the cytoplasm in 5 (6.9%) cases. ΔNp73 protein expression was positive in 48 of 83 (57.1%) ovarian cancer samples. Of these, ΔNp73 was localized in both the nucleus and cytoplasm in 26 (54.2%) cases, but was found in the nucleus in 21 (43.7%) cases and in the cytoplasm in 1 (2.1%) case. Thus, as suggested by Bozzetti et al. (8), both nuclear and cytoplasmic staining in $>10\%$ of tumor cells was considered positive for further analyses.

RT-PCR for *TAp73* and the expression of N-terminally truncated p73 isoforms (*ΔN'p73* and *ΔNp73*) was available in 64 patients, respectively. TAp73 and ΔNp73 protein expression correlated with mRNA quantification in 25 of 64 (39.1%) and 37 of 64 (57.8%) ovarian cancer samples, respectively.

Of the examined 83 primary ovarian cancer specimens, 26 (31.3%) harbored wild-type *p53* and 57 (68.7%) mutant *p53*. TAp73 and ΔNp73 protein expression were not associated with the *p53* mutational status and the well-known clinicopathologic parameters (ie, age, FIGO stage, grading, histologic subtype, residual tumor after primary surgery). In contrast, the *p53* mutational status significantly correlated with tumor grading ($P<0.001$). Twenty-three of 26 (88.5%) *p53* wild-type cancer specimens showed moderate differentiation (grade 2), whereas the majority of *p53* mutant cancer specimens (32 of 57, 56.1%) showed poor differentiation (grade 3).

TAp73 and ΔNp73 protein expression did not influence prognosis in the examined ovarian cancer

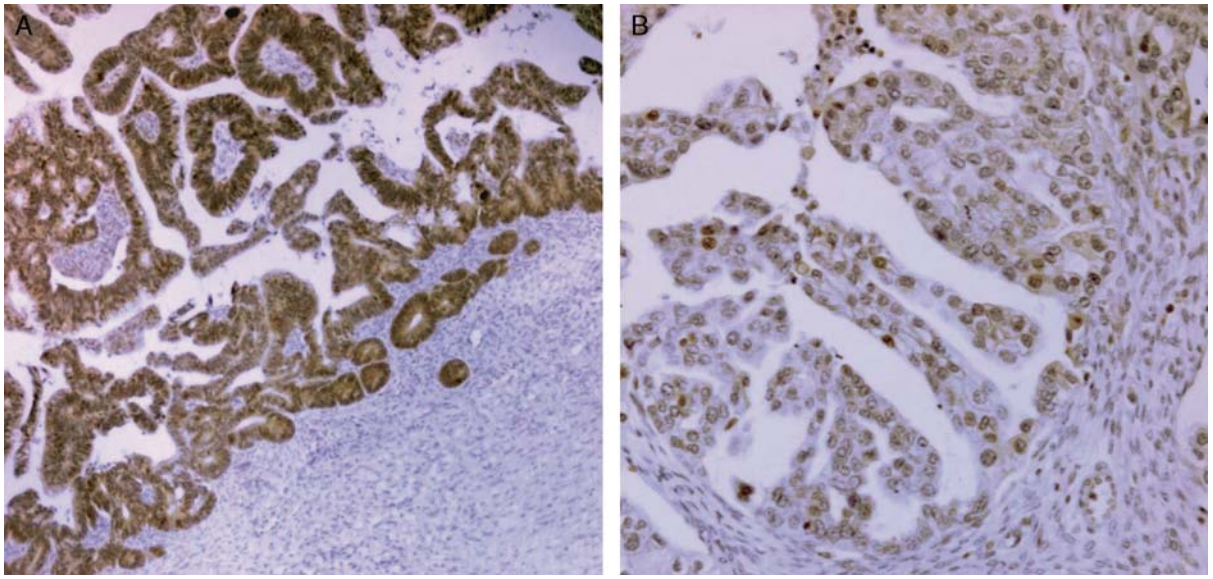

FIG. 1. TAp73 and ΔNp73 protein expression in ovarian cancer specimens. (A) Representative immunostaining for TAp73 localized in both the nucleus and cytoplasm of tumor cells. (B) ΔNp73 staining confined preferentially to the nucleus (original magnification, 20 ×).

cases. Patients with wild-type *p53* cancers had a significantly improved recurrence-free and overall survival in comparison with patients with *p53* mutant cancers (median, 194 vs. 22 mo, $P = 0.020$ and median, 81 vs. 36 mo, $P = 0.039$, respectively). For multivariate analysis, we considered the 4 strongest clinicopathologic prognosticators from the univariate analyses (age, FIGO stage, grading, and residual tumor after primary surgery). The *p53* mutational status was associated with elevated hazard ratios for recurrence-free (hazard ratio, 1.648; 95% confidence interval 0.781–3.476) and overall survival (hazard ratio, 1.706; 95% confidence interval 0.874–3.330), which did not reach statistical significance ($P = 0.189$ and 0.118, respectively).

DISCUSSION

Prompted by our previous study, which had revealed a prognostic significance for N-terminally truncated p73 isoform transcript levels, we aimed to determine the role of TAp73 and ΔNp73 protein expression in ovarian cancer.

Protein methods for the detection of p73 isoforms possess clear limitations. ΔNp73 differs from TAp73 solely by 13 N-terminal residues. To date, only one commercially available monoclonal antibody recognizing this unique sequence has been generated (clone 38C674; Calbiochem and Imgenex, San Diego, CA). In a recent study comparing ΔNp73 antibodies it has proved to be specific for ΔNp73 (8). However, the commercially available ΔNp73 antibody only weakly detected ΔNp73β even in Western blot analyses. Thus, this antibody possibly underestimates the expression of total ΔNp73. Some researchers have used custom polyclonal ΔNp73 antibodies for immunohistochemistry (3,4). Rosenbluth et al. (9), however, reported that their custom ΔNp73 antibodies have not performed consistently in immunohistochemistry.

In this study, the overwhelming majority of ovarian cancer specimens showed TAp73 and ΔNp73 protein expression in both the nucleus and cytoplasm of tumor cells. The nuclear localization is consistent with the function of TAp73 as a transcription factor. It is, however, unclear whether p73 isoforms are functionally active in the cytoplasm *in vivo*. Shuttling from the nucleus to the cytoplasm has been associated with their degradation (10). Interestingly, the C-terminal tail of p53 isoforms has been reported to influence their subcellular localization. p53β has been predominantly found in the nucleus, whereas p53γ has been present in the nucleus and cytoplasm (11).

Previous reports on the subcellular localization of ΔNp73 have been inconsistent. This is not only caused by the usage of different ΔNp73 antibodies. Using the same custom antibody, we observed ΔNp73 immunostaining in both the nucleus and cytoplasm of ovarian cancer cells, whereas Uramoto et al. (4) have reported solely cytoplasmic staining in the overwhelming majority of lung cancer samples. This differing localization of ΔNp73 does not seem to be related to the type of cancer. In breast cancer, ΔNp73 was found exclusively in the cytoplasm in one study and exclusively in the nucleus in another study, using the same commercially available antibody (7,12). Again, these contradictory findings illustrate the questionable reliability of the ΔNp73 antibodies available for immunohistochemistry.

ΔNp73 protein expression lacked prognostic significance in the examined ovarian cancer cases. Previously, we showed that transcript levels of N-terminally truncated p73 isoforms were determinants of recurrence-free and overall survival in patients with *p53* mutant cancers. ΔNp73 is able to inactivate the proapoptotic TAp73 and wild-type p53 protein in *p53* mutant cancers. Of note, TAp73 and ΔNp73 protein expression showed a weak correlation with mRNA quantification in the examined ovarian cancer cases, possibly explaining the conflicting findings related to prognostic significance. In addition to the limitations of immunohistochemistry, posttranslational modifications of p73 isoforms have to be considered. In some previous studies, performing immunohistochemistry, however, ΔNp73 protein expression has possessed prognostic significance in distinct cancer types (2–5).

In conclusion, a potential clinical role of TAp73 and ΔNp73 protein expression cannot be definitely excluded in ovarian cancer using the currently available methods for protein detection.

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