ΔNp63 expression is associated with poor survival in ovarian cancer

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Background: P63 belongs to the ‘p53 family’ whose role in cancer progression has been recently revisited in light of the plethora of splicing variants that are generated. We analyzed the expression of the full-length TAp63 gene and its dominant-negative form ΔNp63 in ovarian cancer biopsies to correlate their expression with clinical outcome.

Materials and methods: Real-time RT–PCR analysis was used to determine the levels of TAp63 and ΔNp63 in 83 stage I and in 86 stage III ovarian cancer biopsies and in seven human ovarian cancer cell.

Results: TAp63 levels were comparable in stage I and stage III, but ΔNp63 levels increased 77-fold in stage III, independently of the p53 status. Patients with high ΔNp63 expression had the worst overall survival (OS); patients with a ΔNp63/TAp63 ratio >2 had a poor OS. Patients with a high ΔNp63/TAp63 ratio were those with a poor response to platinum-based therapy.

Conclusions: Data indicate a role for ΔNp63 as a potential biomarker to predict patient’s outcome and tumor progression in ovarian cancer. This would have particularly clinical relevance in ovarian cancer where the high rate of mortality reflects our lack of knowledge of molecular mechanisms underlying cell progression toward malignancy.

Key words: epithelial ovarian cancer, p53 family, p63

Introduction

P63 (along with p53 and p73) is a well-studied transcription factor belonging to the ‘p53 family’ [1, 2]. The close similarities in their overall structure led to the early speculation that they could act as transcription factors on similar or even identical sets of target genes to that regulated by p53 itself [3, 4]. This high structural homology does not necessarily translate into functional redundancy and p63 and p73 play quite different biological roles, sometimes p53 independent. p53 is inactivated in a majority of human cancers but is largely dispensable for normal development, while Np63 is dispensable for normal development, while inactivated in a majority of human cancers but is largely involved in the plethora of splicing variants that are generated.

In fact, in different cellular models, such as murine embryonic fibroblasts, p63 and p73 are rarely mutated in tumors [5] but more strikingly involved in normal neuronal or epithelial tissue maintenance [6]. The role of p63 in tumorigenesis is still not clear, mainly due to incongruent data on the leading role in driving cell cycle arrest and cell death [7–17]. In fact, in different cellular models, such as murine embryonic fibroblasts, p63 and p73 were reported to cooperate with p53 in driving apoptosis, while in T cells, p63 and p73 are not required to p53-dependent apoptosis and development [18, 19].

One major reason for this uncertainty is that p63 is a complex gene encoding six different isoforms. At present, two different transcription initiation sites have been described for p63: a canonical ‘P1’ upstream promoter from which the full-length transactivation active (TA) protein is encoded, which contains an acidic transcriptional activation domain similar to p53 and the ΔN isoforms, transcribed from a cryptic ‘P2’ promoter within intron 3, which lack the TA domain encoded by exons 2 and 3 but gain 14 unique residues in their N terminus. This scenario is further complicated by the splicing at the 3’ end of the gene, resulting in at least three different COOH terminals α, β and γ; hence, six p63 isoforms are potentially present in cells. Because different isoforms have different and sometimes opposite activities, it is important to identify the expression levels of different p63 family gene isoforms in order to understand their respective function in different stage of neoplastic tissue progression. As a general rule, the TA isoforms are transcription factors sharing many p53 functions such as cell cycle control and induction of apoptosis. The ΔN isoforms potentially act as dominant negative not only on the p63 and p73 but also on the p53 pathway [14, 20, 21]. Recently, however, it has been found that ΔNp63 is endowed with transcriptional activity due to the presence of a second transactivation domain in its N terminus [22, 23]. Amplification and overexpression of TAp63 has been linked to an increase in survival rates in lung cancer patients [24], while its loss has been linked to an increase in...
metastasis in bladder [25, 26] and breast cancer [27]. ΔNp63α is the primary p63 variant expressed in different epithelial tumors and in particular in squamous cell carcinomas of the esophagus, lung, head and neck cervical cancer [28–31]. The amplification of the p63 locus gene on chromosome 3q27–28 may contribute to its disregulated overexpression. The data supported by the notion that ΔNp63 is involved in neoplastic growth [32]. This putative oncogenic role of ΔNp63 isoforms in squamous cell carcinoma is further supported by the notion that ΔNp63 induces proliferation and tumor growth, led to β-catenin-increased accumulation [33] and suppresses p73-dependent apoptotic program [34].

Little is known about how p63 isoforms are involved in ovarian cancer progression. In the present study, the largest to date, we used real-time RT–PCR to measure the absolute copy numbers of TAp63 and ΔNp63 isoform expressions in 169 ovarian cancer biopsies at different stage of diagnosis and we correlate them to tumor progression and prognosis. The results obtained were also analyzed together with the p53 gene status.

**materials and methods**

**cell culture**

Ovarian cancer cell lines, A2780, IGROV-1, SKOV-3, SW626, OVCAR-3, OVCAR-5 and OVCAR-8 were routinely maintained at 37°C, 5% CO₂ in RPMI-1640 medium supplemented with 10% fetal calf serum.

**sample collection**

Biopsies were collected at the Department of Oncology and Gynecology, San Gerardo Hospital (Monza, Italy) from September 1992 to March 2004: 83 were diagnosed as stage I ovarian cancer and 86 as stage III. Their histopathological features are resumed in Table 1. Fresh tumors tissues were obtained at first laparotomy before treatment. The tissues were fresh freed from necrotic, hemorrhagic and connective tissue, minced and kept frozen at −80°C. The collection and use of tumor samples was approved by the local scientific ethical committee, and patients gave their written consent.

**RNA isolation and complementary DNA preparation**

From each frozen sample, a tumor fragment was taken and total RNA purified using SV Total RNA isolation system (Promega, Milan, Italy). The same protocol was used to prepare total RNA from exponentially growing cancer cells. Two hundred nanograms of total RNA was reverse transcribed (High Capacity cDNA Archive Kit, Applied Biosystems, Monza, Italy) and then stored at −80°C until use.

**primer design**

Primer pair sequences are reported in Supplemental Table 1 (available online), while Supplemental Figure 1 (available online) reports the regions where the primers have been drawn. The optimal primer pairs were chosen spanning splicing junctions using PRIMER-3 software available free-share online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers were queried against the nonredundant Human Genome Database (National Center for Biotechnology Information) and the specificity of the PCR products verified by direct sequencing of the amplified amplicons.

**real-time RT–PCR**

Absolute copy numbers were determined by real-time RT–PCR (ABI-7900, Applied Biosystems) with Syber Green protocol (Applied Biosystems). The 384-well reaction plates were prepared by automatic liquid handling (epMotion® 5075 L, Eppendorf, Milan Italy). The absolute copy numbers of targets and housekeeping genes were calculated by interpolation using a standard curve generated from a serial dilution, ranging from 2 to 20 million copies, of a plasmid complementary DNA previously prepared.

**mutational status of p53**

Detection of p53 mutational status in exons 4–9 was carried out in tumor specimens with the following set of primers: forward (p53Fw) 5'-GGGA CAGGCAATGTCGTGACT and reverse (p53Rw): 5'-CCTGGCGCATCC TTGAGTT. Amplification was carried out with 33 cycles at 95°C for 1 min, 60°C for 30 min, 72°C for 1 min and sequenced at local facility (http://www.primm.it).

**data analysis**

Statistical analysis was carried out using the R 2.1.0 software. B-actin, cyclophilin A, glyceraldehyde-3-phosphate dehydrogenase and 28S have been chosen to normalize data. A normalization index has been generated for each patient, computing the geometric mean of the four proposed housekeeping genes. The reliability of the choice of those genes has been evaluated observing the correlations among the genes and between each gene and the normalization index, verifying that an acceptable correlation level exists.

Since the values of gene expression for both stage I and stage III displayed a non-normal distribution, statistical analysis was carried out using a two-tailed Spearman test to evaluate correlations calculated between each patient subset. The Spearman correlation test was also used to evaluate the correlations between TAp63 and ΔNp63 isoforms. P values <0.05 were considered as statistically significant.

**statistical analysis**

The ratio between ΔNp63 and TAp63 (on a logarithmic scale) was analyzed both as a continuous and as a dichotomous variable. The cut-offs used to split the ratio into categories were first the tertiles of the TAp63 and ΔNp63 distributions and then were set to 2. Gaussian distribution of data was tested with the Shapiro–Wilk test for normality; as this test gave a nonsignificant result, a nonparametric analysis of variance was used to evaluate the associations between ΔNp63/TAp63 ratio and categorical variables such as stage, grading, residual tumor and histology. Overall survival (OS) was defined as the length of time from first surgery to the last follow-up date or death irrespective of the cause. OS curves were plotted with the Kaplan–Meier method. The log-rank test and Cox proportional hazards model were used to compare time-to-event distributions between predefined groups. The estimates from the Cox regression model are presented as hazard ratios (HRs) and 95% confidence intervals (CIs).

Three different Cox multivariate models were built to study the prognostic effect of the ΔNp63/TAp63 ratio considered both as dichotomous and continuous variable: the first model analyzed the whole sample of patients and included stage as covariate; the second model analyzed stage I patients and included tumor grade and the third model analyzed stage III patients and included the residual tumor as covariate. Statistical significance was set at 0.05 and all the tests were two tailed. Analysis was carried out using SAS software, version 9.0 (SAS Institute, Inc., Cary, NC).

**results**

A cohort of 169 ovarian cancer biopsies, with a median follow-up of 6.8 years and with a median age at time of diagnosis of 53.5 years, was selected. The relative histopathological...
Table 1. Clinical parameters and chemotherapy regimens of the 169 patients analyzed

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<th>Median age at diagnosis</th>
<th>Histopathological parameters</th>
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<th>DDP</th>
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ND, not detected; CBDCA, carboplatin; DDP, cisplatin; TEP, DDP + epirubicin + cyclophosphamide; Tax, taxol; PAC, DDP + adriamycin + cyclophosphamide; CTX, cyclophosphamide.

Parameters are summarized in Table 1: 50% had International Federation of Gynecology and Obstetrics (FIGO) stage I disease and 50% FIGO stage III disease.

Data reported in Table 1 show that in the examined exons, p53 was wild type (wt) in all stage I. In stage III, we found 47.67% of patients wt for p53 (41 of 86) and 45.35% (39 of 86) harboring mutations. For 7% of them (6 of 86), we were unable to run sequencing protocol. Mutations were more frequent within exon 5 (30%) followed by exons 7 and 8 (25%). Table 1 summarizes the different platinum-based chemotherapeutic regimens both for patients with stage I and stage III diseases. Messenger RNA (mRNA) levels for TAp63 and ΔNp63 isoforms were measured by real-time RT–PCR in all 169 ovarian cancers and in a panel of seven different ovarian cancer cell lines. Primers used in this study were able to discriminate between the NH$_2$-terminal isoforms only while were completely unable to provide information on any of the C-terminal isoforms (Supplemental Figure 1, available online). Absolute copy number value for each isoform was normalized against the normalization index.

When we plotted the absolute mRNA copy numbers of the two isoforms in log scale, stage I (Figure 1) gave a comparable expression profile for TAp63 and ΔNp63 (median value of 3 × 10$^{-3}$ and 5.9 × 10$^{-3}$ for TA and ΔN, respectively, Table 2), while in stage III, the ΔNp63 isoform increased compared with the TAp63 by a median of 77.41-folds (median value of 6.2 × 10$^{-2}$ and 4.8 × 10$^{-2}$ for TA and ΔN, respectively, Table 2). This difference can be further visualized in a subset of seven ovarian cancer cell lines in which the ΔNp63 was more expressed than the TAp63 by a median of 11.48-folds (median value of 3.01 × 10$^{-2}$ and 2.56 × 10$^{-2}$ for TA and ΔN, respectively, Table 2).
The median value of normalized copy numbers for TAp63 and ΔNp63 isoforms in 83 stage I and 86 stage III ovarian cancers and in the seven ovarian cancer cell lines. After normalization of each sample to its own set of housekeeping genes, data are expressed as absolute copy numbers in a logarithmic scale. The line within the boxes indicates the median expression level. The top edge of the boxes represents the 75th percentile and the bottom edge the 25th percentile. The range is shown as a vertical line. Outliers (black circles) are defined as 1.5-fold above or below the 75th and 25th percentile values.

Table 2. TAp63 and ΔNp63 expression levels

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The median value of normalized copy numbers for TAp63 and ΔNp63 are reported for the 169 tumor samples and the seven ovarian cancer cell lines. Ratio is the median value of the ratio between ΔNp63 and TAp63 value.

Mut, mutated.

reported in Figure 1 and Table 2 that the median values for TAp63 were almost comparable between the two different ovarian cancer stages as well as for the in vitro-selected cancer cell lines (median value of 3 × 10^{-3}, 6.2 × 10^{-3} and 3.01 × 10^{-3} for stage I, stage III and ovarian cancer cell lines, respectively).

As reported in Table 2, ΔNp63 expression levels were not affected by p53 mutational status (5.7 × 10^{-1} and 5.2 × 10^{-1} for p53 wt and p53 mutational subset, respectively) while we measured a drop in the TAp63 expression levels in those patients harboring p53 mutations compared with the wt subset (1 × 10^{-2} and 4.5 × 10^{-3} for p53 wt and p53 mutated, respectively). This finally results in a ΔNp63/TAp63 ratio that is 2.6-fold higher in tumors with p53 mutational subset compared with those with a p53 wt.

For each patient, we analyzed the correlation levels between TAp63 and ΔNp63 according to the different FIGO parameters. As reported in Supplemental Table 2 (available online), the best results, in terms of rho and P value, were observed with stage IIIC and grade 3, which were almost the most representative one.

The ΔNp63/TAp63 ratio was found to be strongly associated with stage (P < 0.0001), grading (P = 0.0021), histology (P < 0.0001) and residual tumor (P < 0.0001) (Supplemental Table 3, available online). Median ΔNp63/TAp63 ratio was 2.18 in stage I (range 0.01–242.79) compared with 65.68 in stage III (range 0.08–2700); patients with borderline or grade 1, 2, 3 diseases had a median value of ΔNp63/TAp63 ratio, respectively, of 1.65 (range 0.01–242.79), 1.82 (range 0.05–204.25), 9.06 (range 0.08–591.40) and 27.75 (range 0.17–2700). As regard to the histotype, the relative limited number of samples of each individual subtype did not allow us to compare in appropriate way the expression level. We, however, noted that comparing the most frequent subtype (serous) versus all the others (mucinous, endometroid, clear cells and undifferentiated), the ΔNp63/TAp63 ratio was higher in serous compared with nonserous tumors (20.32 with a median range of 0.01–2700). Even if clinically clear cells, endometroid and mucinous tumors are well distinct from each other, this comparison highlights the fact that serous tumors type do have a high ΔN/Tα ratio. Furthermore, median value of ΔNp63/TAp63 ratio was smaller in patients with residual tumor <2 cm as compared with patients with residual tumor >2 cm (4.09, range 0.01–242 versus 87.44, range 0.08–2700, respectively).

Survival analysis was carried out in 168 patients, 65 of whom had died (38.7%). We correlated the absolute expression levels of TAp63 with OS plotted with the Kaplan–Meier method. We found no difference in OS between patients with high and low levels of expression of TAp63 (P = 0.58, Figure 2A and Supplemental Table 3C, available online). A significative level of correlation was achieved in stage I (P = 0.0188) where patients with low levels of expression of TAp63 had a worst prognosis than that with high levels. This observation
High levels of ΔNp63 were, however, associated with poor OS ($P=0.0007$, Figure 3A). After 3 years of follow-up, the OS for patients within the second tertile was closed to 69% while for those within the first tertile the OS was closed to 82%. This difference was more consistent after 5 and 7 years of follow-up (Supplemental Table 3C, available online).

Unfortunately, when we tried to correlate ΔNp63 levels with OS in patients with stage I or in stage III separately, we did not reach the significance ($P=0.2$ and 0.9 for stage I and stage III, respectively, Figure 3B and C). The power test indicated that a cohort of at least 230 patients should be analyzed.

Due to the fact that ΔNp63 protein acts in a dominant-negative fashion on the TAp63 network, we tested the hypothesis that patients with a ΔNp63/TAp63 ratio different from that measured in stage I (i.e. 2) could have a different clinical outcome. ΔNp63/TAp63 ratio was analyzed as
Figure 4. Kaplan–Meier survival curves in relation to ratio between ΔNp63 and TAp63 expression in all patients (A) and in stage I (B) and stage III (C) subpopulations. Patients were divided in those with a log ratio ΔNp63/TAp63 <2 and those with a log ratio ΔNp63/TAp63 >2.

In the present paper, we analyzed the expression levels of TAp63 and ΔNp63 epithelial ovarian cancer (EOC), which has one of the worst prognosis among gynecological cancers in the Western world [35]. We found that both TAp63 and ΔNp63 were expressed at comparable levels in stage I while in the stage III we observed a clear and systematic change in the ΔN/TA ratio due to a marked increase in the ΔNp63 levels only. High ΔN/TA ratio has been correlated to a reduced OS in EOC patients, thus supporting for the first time a fundamental role for ΔNp63 in EOC progression.

A previous study in a cohort of 100 ovarian cancer biopsies showed that deregulation of the P1 promoter rather than P2 up-regulation is the main event which drives p73 overexpression in tumor specimens compared with normal counterparts [36]. Additionally, NH2-terminal-truncated p73 isoforms contribute to drug resistance in patients harboring p53 mutations, thus representing an addition unfavorable factor to response to platinum-based therapy [37]. Interestingly, TAp73 levels were, in those patients and in a similar study conducted in our laboratory, always higher than those of ΔNp73 [38]. In the present study, we found not only that ΔNp63 expression strongly increases in patients from stage I to stage III but that already in early ovarian cancer patients the levels of ΔNp63 are higher than those of TAp63. This would imply that ΔNp63 can play an important role in tumor progression. The finding that in our subset of patients, ΔNp63 overexpression and p53 mutations are not related to each other [31], indicates that in this context, ΔNp63 oncogenic role is independent of its transdominant activity on the p53 pathways and could be related to its own transcriptional activity properties.

Although this was beyond the scope of this work, we tried to determine whether there was any association between ΔN/TA p63 levels and response to chemotherapy. According to the RECIST criteria a complete response (CR) was defined as the disappearance of all target lesions; a partial response (PR) as the 30% decrease in the sum of the longest diameter of target lesions; a progressive disease (PD) as the 20% increase in the sum of the longest diameter of target lesions while a stable disease (SD) when less than a 30% reduction and less than a 20% increase in the sum of the longest diameter of target lesions and the appearance of no new lesions. In our dataset, response to chemotherapy was available for 121 out to 179 patients; 95 had CR or PR and 26 had SD or PD. The ΔN/TA ratio was 43 for CR + PR and 82 for SD + PD. These data need to be confirmed in a larger study designed specifically to address this point, but it indicates that a higher ratio of ΔNp63 to TAp63 is associated not only with OS but also with response to treatment. These data provide the first evidence of a fundamental role for ΔNp63 in EOC, which has one of the worst prognosis among gynecological cancers in the Western world [35].
progression since higher ΔNp63 levels were correlated with shorter OS and poor response to therapy.

The idea that ΔNp63 might be related to EOC progression could thus serve as a putative biomarker for advanced and malignant grades in line with reports in which the ΔNp63α isoform is required to proliferate and maintain the proliferative potential in tumor tissue [39, 40]. The proliferative potential role of ΔNp63 is also supported by our finding that seven different in vitro exponentially growing cancer cell lines were characterized by an increased, even if not comparable to clinical data, expression levels of ΔNp63 isoform compared with the TAp63 one.

In conclusion, the overall data from this study support the idea that within the p53 family, the balance between the TAp63 and ΔNp63 isoforms may have an impact on EOC progression.

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