Work in progress report - Experimental

Expression of cyclins D1, D3 and p27 in thymic epithelial tumors

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Received 6 July 2003; received in revised form 22 October 2003; accepted 30 October 2003

Abstract

In this study, the expression of cyclins D1 and D3, as well as cyclin-dependent kinase inhibitor p27 in thymic epithelial tumors (thymomas) is examined. Histological specimens from 24 patients (11 males and 13 females) were submitted to classification according to WHO criteria. Staining for cyclins D1, D3 and p27 was applied and evaluation was performed for expression of D1, D3 and p27. Eighteen patients presented low-grade thymomas (nine B1, predominantly cortical; three B2, cortical; six B3, well-differentiated thymic carcinoma) and six patients benign thymomas (four A-medullary, two AB-mixed). The p27 expression in patients with benign thymomas was 42\%\textsuperscript{26}, whereas in patients with low-grade thymoma, it was 11\%\textsuperscript{20}. The expression of cyclins D1 and D3 was 2.8\%\textsuperscript{2.7} and 10\%\textsuperscript{6} for benign as well as 8.3\%\textsuperscript{9.6} and 12\%\textsuperscript{10} for low-grade thymomas, respectively. A statistically significant difference was revealed regarding the p27 expression through different grades (analysis of variance $P$-value 0.00076) and histopathological types of thymomas ($P = 0.0047$). This finding of greater p27 expression in benign thymomas with progressive reduction in higher grades is compatible with observations on other soft tissue and solid tumors suggesting that p27 level decreases during tumor development and progression.

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Keywords: Cyclin-dependent kinase; Cyclin-dependent kinase inhibitors; Thymus; Epithelial tumors; Cell cycle

1. Introduction

Cyclins and cyclin-dependent kinases (CDK) have been identified to have a central position in main control points of the cell cycle, which is recognized as a critical determinant of oncogenesis.

CDK activity is subjected to negative control by two mechanisms: binding of inhibitory subunits and phosphorylation on an amino-terminal residue.

Two classes of CDK-inhibitory proteins (CKI) negatively regulate the cell cycle by binding to and inhibiting CDK: the INK4 proteins (p15, p16, p18, and p19), which specifically inhibit the CDK4/6 kinases and the Cip/Kip proteins (p21, p27, p57), which react with a broad range of CDK [1].

Cyclin D1 is implicated in several tumors such as B-cell lymphomas and parathyroid adenomas [2]. Cyclin D1 overexpression has also been reported in mantle cell non-Hodgkin lymphoma, in 15% of prolymphocytic and 6% of chronic lymphocytic leukemias and in other solid tumors [3]. Regarding the role of cyclin D3 in oncogenesis, overexpression of the cyclin has been associated with proliferative activity and tumor progression in non-Hodgkin lymphomas [4,5].

On the other hand, an inverse correlation has been revealed between tumor progression and p27 expression as decreased p27 expression has been described in aggressive breast, gastrointestinal, lung carcinomas, non-Hodgkin lymphomas and melanomas [6].

Although the expression of cell cycle regulators has been investigated in normal human and mouse thymus [7], the role of cyclins D and p27 has not been previously studied in thymic epithelial tumors (TET). The term TET has been adopted instead of thymomas according to recent histological classification [8] in order to distinguish tumors derived from the epithelial component of the thymus from tumors originating from the other cell lines of the gland.

The aim of this study is to evaluate the expression of cyclins D1 and D3 as well as CKI p27 in the epithelial component of TET.
2. Materials and methods

2.1. Patients

During a 5-year period between 1995 and 1999, 24 patients were managed surgically with radical resection of TET in the Thoracic Surgery Department of ‘Evangelismos’ General Hospital. Radical resection was a prerequisite for the introduction of a patient in the study and included adjacent mediastinal adipose tissue consisting thymus gland and perithymic tissue. All patients (11 males and 13 females), aged 17–80 years old (mean 54.1/median 55 years) were studied in reference to the expression of p27 and cyclins D1 and D3. Diagnosis of TET was implicated preoperatively from radiographic appearance; it was an incidental finding in nine patients without myasthenia gravis (MG), whereas four patients without MG presented dyspnea, dysphagia, cough and pleuritic pain. In the remaining 11 myasthenic patients, TET was discovered in the investigation of MG. All patients had been submitted preoperatively to computed tomography (CT) of the chest and examination for myasthenic antibodies. Definite histological confirmation was achieved with the resection of the tumor. Preoperative fine-needle aspiration or core-needle biopsy was not performed because of the risk of tumor spillage. Two reviewers (DR and KS) reassessed the histology of the material independently and without previous knowledge of the specimens and classified the tumors according to the new World Health Organization (WHO) classification system [9]. Table 1 has the clinical and histological features of the patients at the time of the operation. The type of the TET, Masaoka stage and presence of MG are signaled in this table.

2.2. Surgical technique

As mentioned above, a technique for resection of all thymic and perithymic soft and fatty tissue with TET was applied via median sternotomy. A transverse cervical incision was never opened, unless invaded from the mass, and mediastinal pleura were not routinely incised, unless surgical manipulations were to be facilitated. The left phrenic nerve was always identified and preserved as well as the recurrent laryngeal nerve at the aortopulmonary window. Two drains were inserted for the mediastinum and the pleural cavity if opened.

2.3. Technique

Formalin fixed and paraffin-embedded tissue from the patients was classified according to the WHO classification system. The p27 expression was investigated on paraffin-embedded tissue sections with the monoclonal antibody (mab) anti-p27 CALBIOCHEM 1:20, cyclin D3 expression with the mab anti-cyclin D3 DCS22 DAKO 1:20, and cyclin D1 expression with the mab anti-cyclin D1 DAKO 1:20. DR and KS reviewed all immunohistochemical analyses. Immunohistochemical staining in one representative section of the specimen was performed on an automated immunostainer (Techmate-DAKO) according to the company’s protocol.

Double staining for p27 and MNF-116 cytokeratin, cyclin D1 and MNF-116 cytokeratin, as well as for cyclin D3 and MNF-116 cytokeratin, were performed using two different detection systems. The primary antibody p27 or cyclins D1 or D3 was incubated overnight and the secondary antibody for 30 min. The reaction was developed using the ABC complex and 3,3-diaminobenzidine. The second primary antibody MNF-116 was incubated for 1 h and the secondary one for 30 min. The reaction was then detected with an ABC-alkaline phosphatase complex.

Positive controls for p27, cyclins D1 and D3 were used to confirm the adequacy of staining. The staining quality of cyclins D1 and D3 was considered valid when epithelial cells and histiocytes exhibited characteristic weak nuclear positive staining. Staining was not performed in two patients for cyclin D1 and in five patients for cyclin D3 and these patients were excluded from the statistical analysis. Lymphocytes were used as internal controls to compare p27 staining between tissue samples. The percentage of cells showing positive nuclear staining and cytoplasmic cytokeratin was assessed for p27, cyclins D1 and D3, respectively, in each case via a semi-quantitative method of measurement. A grid ocular objective was used to count 400 cells over three high-power fields (×40) and the percentage of positive cells was reported as 0–100%.

2.4. Statistical analysis

Results were reported as mean ± SD, standard error, and confidence limits. Between group comparisons were performed with one-way analysis of variance to assess the overall statistical significance. Individual comparisons between different histological types of benign (A, AB) and low-grade (B1, B2 and B3) TET were performed using...
Tukey’s honestly significantly difference (HSD) test for unequal sample sizes.

3. Results

3.1. p27 expression

The immunohistochemical expression of p27 in patients with benign and low-grade TET was 42 ± 26 and 11 ± 13%, respectively. These findings represent a statistically significant difference (analysis of variance $P = 0.00076$) of expression between the two groups (Table 1). The detailed analysis of subgroups in low grade and benign TET showed a progressive decrease of expression from subtype A to B3 (Figs. 1 and 2). This decrease of expression is documented as statistically significant (analysis of variance $P = 0.0047$), as p27 expression is gradually reduced in parallel with more aggressive subtypes of TET (Table 1).

No relation was documented between the p27 expression and Masaoka staging. Paradoxically, a high p27 expression rate was observed in stage III (mean 37%), with increased SD (±46%) in a sample of extremely limited power (two patients). The p27 expression in the other two stages showed no significant difference as level of detection are 23 ± 22.5% for stage I, and 14 ± 17% for stage II.

3.2. Cyclin D1

Cyclin D1 showed a low expression in all subgroups and grades of TET. Although there is no significant correlation between cyclin D1 expression and grade, a trend for increased cyclin D1 expression in parallel with tumor grade was observed. Cyclin D1 expression started from 2.5 ± 2.9% in subtype A of benign TET and reached 10 ± 9% in subtype B3 (Table 2).

No relation was documented between D1 expression and Masaoka stage.

3.3. Cyclin D3

As for cyclin D3, no consistent pattern of expression was observed so as to document any correlation. No significant difference was documented between the D3 expression in low-grade TET (12 ± 10%) and its expression in benign TET (10 ± 6%). Moreover, cyclin D3 showed no significant correlation with Masaoka stage.

Such an inconsistent pattern of the cyclin D3 expression was not possible to result in any correlation of cyclin D3 with histological type and grade.

4. Discussion

Cyclins are a family of key cell-cycle regulators that, activated with CDK, phosphorylate various proteins, which are important for the cell-cycle progression. D-type cyclins (D1, D2, and D3) are expressed in the G1-phase of the cell cycle by forming complexes with and activating CDK 4 and CDK 6 [10,11].

Cyclins, CDK complexes and CKI, especially those controlling the G1/S phase transition of the cell cycle, are frequently deregulated in human malignancies [11–13]. Overexpression of D-type cyclins (D1, D2, D3) seem to play an important role in the oncogenesis of human tumors, while decreased levels of p27 are observed in a variety of human malignancies [1,6,12].

<table>
<thead>
<tr>
<th>Grade</th>
<th>No. of cases</th>
<th>Cyclin D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>6</td>
<td>0.028 ± 0.027</td>
</tr>
<tr>
<td>Low grade</td>
<td>16</td>
<td>0.083 ± 0.096</td>
</tr>
<tr>
<td>Analysis of variance, $P = 0.16$</td>
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The p27, as CKI, belongs to the second group of CKI, the Cip/Kip family, and its expression has the character of a tumor suppressor gene. Loss of the p27 protein expression may result in tumor development and/or progression [6]. Applying a semi-quantitative method of measurement p27 expression showed a significantly lower expression (11%) in low-grade TET than in benign TET (42%). It is known that p27 level decreases during tumor development and progression in some epithelial, lymphoid, and endocrine tissues. The same pattern is repeated in TET, as a gradually decreasing p27 expression is observed from benign to more aggressive TET. A known exception to this model of expression has been uniquely reported in very few distinct, rather aggressive non-Hodgkin’s lymphoma entities where shortened survival seems to correlate with high expression of p27 [1].

The p27 increased expression in A (medullary) and AB (mixed) TET reflects its expression in normal human thymus, as p27 expression is undetectable in normal subcapsular thymocytes with a trend for increased expression toward the normal medulla [7].

Cyclin D1 showed low expression in all subtypes of TET with a trend for higher expression in low-grade TET than in benign ones while cyclin D3 showed an inconsistent pattern of expression with no significant correlation with grade or stage. Expression of cyclins D1 and D3 has been previously reported in mouse and human normal thymus [7,14]. A weak cyclin D1 expression was observed in mouse thymus cells, whereas cyclin D3 gene was dominantly expressed in thymus and spleen [14]. The limited cyclin D1 expression in our TET may reflect the low detection in human thymus [7], whereas the inconsistent pattern of expression of cyclin D3 needs further investigation.

All in all, a principal role could be suggested for p27 as CKI in TET. The cell cycle seems to have this inhibitor as a key factor in its regulation. Until now, a number of studies have characterized p27 as an independent prognostic factor in various human cancers, such as breast, colon, and prostate adenocarcinomas. TET is potentially to be added to this tumor series. However, according to the Steeg and Abrams criteria, in order to introduce a new prognostic factor into routine clinical use, at least three criteria must be met: (1) a marker providing information independent of and better than conventional pathological criteria; (2) a marker producing information that can alter treatment decisions; (3) a reproducible marker in studies [15].

In conclusion, the findings of this study suggest that a significant correlation exists between p27 expression and tumor grade. Whether this also means different therapeutic strategies and whether it will be confirmed in new studies remains to be seen.

**Acknowledgements**

The authors would like to thank Drs Antonios Loutsidis and Antonios Chatzimichalis as active members of the staff in the Department of Thoracic Surgery of Evangelismos Hospital, as well as Prof. Theodosios Dosios for his valuable comments and remarks.

**References**