**Abstract.** Aim: To investigate the protein kinase B (Akt) signalling proteins phosphatase and tensin homolog (PTEN), phosphorylated-Akt (p-Akt) and cyclin-dependent kinase inhibitor 1B (p27\(^{\text{Kip1}}\)) in non-seminomatous germ cell tumors (NST) with a view to future investigative approaches. Materials and Methods: The expressions of PTEN, p-Akt and p27\(^{\text{Kip1}}\) were immunohistochemically assessed in 17 teratomas, 27 embryonal cell carcinomas, 6 yolk sac tumors and 24 benign testicular parenchymas. The cytoplasmic and corresponding nuclear expressions were compared and correlated to tumor entity. Results: PTEN was dramatically reduced in all the NST subgroups. Concentrated nuclear p27\(^{\text{Kip1}}\) and loss of the cytoplasmic form was found in teratomas and embryonal cell carcinomas. Neither altered expression nor negative Akt regulation was found. The yolk sac tumors showed late cytoplasmic shift of PTEN and p27\(^{\text{Kip1}}\). Conclusion: Both, the absence of overexpression of p-Akt and of negative correlations to PTEN and p27\(^{\text{Kip1}}\) suggest that signalling of these parameters in NST might include additional mechanisms such as crosstalk to other pathways rather than classical Akt activation.

The prognosis of germ-cell tumors has improved tremendously since the introduction of cisplatin based therapy regimens, especially in metastasized non-seminomatous tumor (NST) patients. Nevertheless some patients are insensitive to cisplatin or a subsequent high dose chemotherapy regimen. Such cisplatin insensitivity significantly worsens their prognosis and decreases their risk of cancer-specific survival. The remaining therapeutic options are limited (1).

Molecular therapy, directly targeting specific signalling cascades has been successfully introduced into the clinical routine of several solid tumors (2). For testicular cancer, these new agents are or have been applied only in a few phase I/II studies in poor prognosis patients, which failed standard chemotherapy (3). So far molecular data and an insight into tumor cell deregulation within these signalling cascades are rare. The analysis of cellular cascades and the respective upstream and downstream parameters may give a new view of the underlying molecular changes in NST and helps to evaluate the possible impact of these therapies in ongoing phase I/II trials.

One of the most frequently targeted cell signalling cascades of tumor cells is the protein kinase B (Akt) cascade with its downstream mammalian target of rapamycin (mTOR) system and of the effector parameters of the cell cycle and proliferation. In urological malignancies the receptor - Akt - axis is a major focus of therapy, for example in renal cell carcinoma, tyrosine kinase inhibitors directed against vascular endothelial growth factor (VEGF) and other growth receptors significantly improved patient survival (4). PTEN, p-Akt and p27\(^{\text{Kip1}}\) have been determined to be common key proteins of Akt signalling and of potential biomarker relevance. Based on the work of Di Vizio et al. (5) on germ cell neoplasias, PTEN and p27\(^{\text{Kip1}}\) seem to be promising candidates to be investigated in conjunction with p-Akt.

The aim of this pilot study was to investigate alterations in and relationships between the PTEN, p-Akt and p27\(^{\text{Kip1}}\) proteins in non-seminomatous germ cell tumors and to give an overview of NST conditions with special considerations of cellular distribution and potential biomarker suitability. Expected results from NST may provide further information which might help to direct research into potential Akt targeting molecules.
Materials and Methods

Patients and histological assessment. Seventy four paraffin tissue samples from 48 men (median age 27.5 years), who had undergone inguinal orchectomy between 09/1993 and 03/2003 at the Department of Urology, University of Tuebingen, Germany were included in this study. The samples were all re-evaluated and graded according to the American Joint Committee on Cancer TNM classification by a pathologist (UV). The cohort comprised 24 benign testicular parenchymas and 50 non-seminomatous germ cell tumors of pure or mixed constellation (6 yolk sac tumors, 27 embryonal cell carcinomas and 17 teratomas). TNM was as follows: T1: 26, >T1: 14 and Tx: 8; N0: 25, N+: 14 and Nx: 9; M0: 31 and M1: 17. The study was approved by the local ethics committee (No. 138/2006).

Immunohistochemistry. For the analysis of mixed tumor types the respective areas were circled in different colors on hematoxylin-eosin stained slides. Specific staining was performed manually with commercially available antibodies against PTEN (monoclonal mouse, dilution 1:100), p-Akt (polyclonal rabbit, 1:50, both Cell Signalling Technology, Inc., Danvers, MA, USA) and p27kip1 (monoclonal mouse, 1:200, Dako Cytomation, Glostrup, Denmark). The tissue slides were incubated overnight at 4°C with the corresponding antibody solutions, then incubated with a secondary biotinylated antibody (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 60 minutes. Visualization was achieved by the DAB method according to the manufacturer's instructions. After brief rinsing, counterstaining was performed with Mayer's hematoxylin and the slides were mounted. Human breast carcinoma served as the positive control for all the antibodies used; for negative control the primary antibody was omitted on breast carcinoma slides. The staining intensity of nuclear and cytoplasmic localization was evaluated in a blinded fashion by two independent investigators. Staining was given as a semi-quantitative score, evaluating the percentage of positive cells multiplied by the respective staining intensity (0-III) as previously described (6) and recently applied in urological cancer (7).

Statistical analysis. All the results are given as mean±SEM. Differences in nuclear and cytoplasmic expression between the tissues were analysed by one way ANOVA with Student’s t-test as post hoc analysis between all pairs. Nuclear and corresponding cytoplasmic expressions of the cohorts were compared by Wilkoxon-Kruskall-Wallis tests and linear regression analyses. JMP 9.0 (SAS Inc., Cary, NC, USA) software was used for the analyses; a p-value <0.05 was defined to be significant.

Results

Representative staining is shown in Figure 1. In the benign testicular parenchyma predominantly nuclear PTEN staining was found, but noticeable cytoplasmic expression was seen as well. Staining was distributed similarly within all levels of the tubuli. Staining of p-Akt was predominantly nuclear with clearly weaker cytoplasmic expression. All levels of the tubuli showed p-Akt expression, however staining appeared to be stronger in the basal levels. The testicular parenchymas showed similar p27kip1 staining at all levels of the tubule wall homogenously, with a predominantly nuclear and minor cytoplasmic subcellular localization. All the investigated malignant NST subgroups showed homogeneously distributed staining of all three parameters.

Expressions of PTEN, p-Akt and p27kip1. One way ANOVA revealed significant differences for cytoplasmic and nuclear expressions of PTEN (both, p<0.001) and cytoplasmic (p<0.0001) and nuclear (p<0.05) expressions of p27kip1 between the tissue entities. Nuclear expression of p-Akt showed no intergroup differences while cytoplasmic expression showed variation (p=0.08).

Comparisons between tissue entities showed significantly lower cytoplasmic expression of PTEN and p27kip1 in the yolk sac tumors, embryonal cell carcinomas and teratomas compared to the benign parenchyma (PTEN: 2.4 [4%], 2.6 [5%] and 12.0 [21%] vs. 56.0 [100%]; p27kip1: 17.5 [21%], 26.4 [32%] and 15.7 [19%] vs. 83.6 [100%], all p<0.001). Nuclear expression of PTEN was significantly lower in all three tumors compared to the benign tissue (1.2 [2%], 1.3 [2%] and 8.7 [15%] vs. 56.6 [100%], all p<0.001), whereas the nuclear expression of p27kip1 was significantly reduced in the yolk sac tumors (34.5 [33%] vs. 105.6 [100%), p<0.02) and embryonal cell carcinomas (65.6 [62%], p<0.05) compared to the benign tissue, but not in the teratomas (93.7). No significant difference was detected for p-Akt expression in the malignant tissue compared to the normal testis parenchyma; except for the yolk sac tumors which showed a significantly lower cytoplasmic p-Akt expression (1.3 vs. 32.3, p<0.02) in the post hoc analysis (Figure 2).

Comparison of cytoplasmic and nuclear localization. The expression scores of cytoplasmic localization are given as the relative percentage of the respective nuclear staining in Table I. The benign testicular tissue showed a slightly lower, but not significantly different, cytoplasmic expression for PTEN, p-Akt and p27kip1. Embryonic cell carcinoma showed a significantly increased expression of cytoplasmic PTEN compared to nuclear expression. The relative cytoplasmic expression (compared to nuclear) of p-Akt and p27kip1 was decreased, which was significant in the embryonal cell tumors and especially in the teratomas.

| Table I. Relationship (cytoplasmatic score as a percentage of the nuclear score/p-value) of cytoplasmic staining to nuclear staining. |
|---------------|---------------|---------------|---------------|
|               | BEN           | YOLK          | EMB           | TER           |
| PTEN          | 99/0.82       | 200/0.58      | 200/0.0184    | 111/0.91      |
| p-Akt         | 69/0.35       | 55/0.75       | 52/0.0074     | 26/0.0001     |
| p27kip1       | 79/0.31       | 51/0.22       | 40/0.0003     | 18/<0.0001    |
In the normal testicular tissue the nuclear expression of all three investigated parameters correlated significantly positively to the respective cytoplasmic expression (PTEN, p<0.01, p-Akt p<0.05 and p27kip1 p<0.001; Figure 3). No positive correlation between nuclear and cytoplasmic expression was detected in the yolk sac tumors and teratomas. In the embryonal cell carcinomas a positive correlation was observed only for p-Akt (p<0.005) and p27kip1 (p<0.001).

**Correlations between PTEN, p-Akt and p27kip1 expressions.** Neither inverse correlation between PTEN and p-Akt expression nor between p-Akt and p27kip1 expression was observed in any of the tissues. In the benign tissue a positive correlation between the nuclear PTEN and p27kip1 expressions was seen (p<0.05).

**Correlations of PTEN, p-Akt and p27kip1 expressions to TNM stage.** The staining scores of the different tumor entities were correlated to the patients’ data: T1 vs. T>1, N0 vs. N+ and M0 vs. M1. Only yolk sac tumors showed clear trends. The cytoplasmically located expression of all three parameters demonstrated alterations in the higher T, N and M stages. Whereas PTEN and p27kip1 showed stronger cytoplasmic expressions, the p-Akt expression was found to be lower in the cytoplasm.

**Discussion**

In all three NST subgroups a dramatic loss of PTEN and a reduced expression of p27kip1 were observed, which was concordant with the common opinion of Akt signalling, where the loss of PTEN has been reported to occur widely in malignant disease leading to activation of the Akt pathway, which results in diminished function of p27kip1 and increased cell proliferation (8).

In NST it has already been reported that the loss of PTEN is involved in the formation of teratoma and that PTEN is associated with germ cell dedifferentiation, which in turn affects p27kip1 expression (5, 9). In the present study the nuclear form of p27kip1 was differentially down-regulated between the subgroups and the teratomas were the least affected (Figure 2) and the nuclear p27kip1 was reduced less than PTEN. These findings were in line with Bartkova et al. (10), who reported (‘unexpected’) sustained expression of p27kip1 in embryonal cell carcinomas and even more in teratomas. Additionally, in the NST tissue the expected increase of the Akt protein was not observed. The expected inverse relationship of PTEN to p-Akt and p-Akt to p27kip1 were also not observed, but due to the pilot character of the present study, the interpretation of these results has to be cautious. Recently, direct association between PTEN and p27kip1 has been reported for several tumors including renal cell (11) and urothelium carcinomas (12) whereas the present study, such association was clearly seen only in the benign parenchyma. Taken together these findings strengthen the suggestion, that other signalling pathways, which bypass the Akt axis are involved in NST tumor biology.

The subcellular localization of PTEN is important in cell physiology (13) and translocation of PTEN from the nucleus to the cytoplasm is associated with malignant diseases (14). In a recent study however, the cytoplasmic shift of PTEN resulted from rather than its caused Akt activation (15). The present loss of PTEN in the NST seemed to affect both compartments equally (Figure 2). Recently nuclear PTEN was shown to lead to p53 mediated growth arrest, while PTEN in the cytoplasm negatively regulated Akt signalling (16). Based on this data, PTEN reduction in NST should be discussed in connection with the p53 protein function, especially, as p53 alterations occur frequently in germ cell tumors (17).

The nuclear accumulation of p27kip1 was evident in the embryonal cell carcinomas and teratomas, in contrast to the phenomenon of a nuclear-export, reported first in breast cancer (18), it was reported to be important in several other human carcinomas (19, 20) and to be at least partly caused by Akt activation (21). However, a different signalling sequence within the dedifferentiation of NST may be involved. The cytoplasmic localization of p27kip1 may be a late event in tumor life, which is supported by the frequent finding that the cytoplasmic form of p27kip1 is of positive prognostic significance (22). In the yolk sac tumors cytoplasmic PTEN and p27kip1 were associated with T as well as N and M status, as was also lower cytoplasmic p-Akt, which may suggest a late-event translocation of PTEN and p27kip1 and nuclear concentration of p-Akt in these tumors.

Therapeutically several aspects should be considered. Acting downstream of the stem cell growth factor receptor (c-Kit), the Akt pathway has been reported to play a role in the development of germ cell tumors (23), but this cascade seems to occur predominantly in seminomas (88% c-Kit+ vs. 44% in NST (24)), suggesting some different mechanisms in seminomas than in NST. The T-cell leukemia/lymphoma 1 (TCL1) protein activates the Akt signalling cascade and has been suggested as a potential therapeutic agent (25). However, in a phase II study with Akt targeting tyrosine kinase inhibitors (26) showed only limited success in cisplatin-resistant germ cell tumors (88% NST); and response rates were marginal (3, 26). If inhibitors of Akt do not improve therapy outcome, then agents directed against up-stream (PTEN) or downstream (p27kip1) located signalling proteins may be of potential therapeutic value. Some evidence suggests that in NST, PTEN loss results in crosstalk deregulation of adjacent pathways such as the mitogen-activated protein kinase (MAPK)/signal transducer and activator of transcription (STAT) signaling cascades (27). The present findings should be confirmed with more samples than this size-limited pilot study.
Figure 1. Representative immunohistochemical staining results. BEN: benign testicular tissue; YOLK: yolk sac tumor; EMB: embryonal cell carcinoma; TER: teratoma, bar: 100 μm, magnification: ×160.

Figure 2. Mean staining scores of subgroups for PTEN, p-Akt and p27Kip1 (Staining score: percentage of positive cells × staining intensity). Significance levels of the Student t-test for statistically different pairs are shown.
In conclusion NST show considerable alterations in Akt signalling, especially for the proteins PTEN and p27Kip1, however a clear pattern of Akt activation is not evident and differences between the tumor subgroups are visible. The cellular deregulation involving crosstalk to other pathways bypassing Akt should be considered. The findings of this first overview pilot study may contribute to the successful search for new therapies for NST.

Acknowledgements
The Authors thank Hannes Schramm for his graphical assistance.

References