**Prognostic Significance of Polo-like Kinase (PLK) Expression in Squamous Cell Carcinomas of the Head and Neck**

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**Abstract**

Previously, we demonstrated that the mammalian polo-like kinase (PLK), which participates in the regulation of the cell cycle, is a novel marker of cellular proliferation. Because current prognostic tools for the evaluation of patients with head and neck squamous cell cancer (HNSCC) need to be improved, we analyzed 89 patients and found elevated PLK expression in most tumors. Nodal stage as a crucial prognostic factor in HNSCC also correlated to PLK transcript levels ($P = 0.0043$). A Kaplan-Meier analysis showed that HNSCC patients with moderate versus high PLK expression survived significantly longer (5-year survival rates, 43% versus 12%; $P = 0.0047$). Interestingly, a combination of nodal stage and PLK expression contributed to discriminate patients with a better prognosis in the $pN_{0+}$ and $pN_{2+}$ groups, which could improve the definition of a suitable therapy.

**Introduction**

More than 500,000 new cases of SCCs of the upper aerodigestive tract are observed annually worldwide. Approximately 90% of these tumors attack individuals who smoke tobacco and/or ingest alcohol. More than 60% of patients apply to hospitals in advanced stages (Union International Contre Cancer stages III and IV). Despite multimodal treatment strategies (surgery, radiation therapy, and chemotherapy), the overall 5-year survival rate in stages III and IV is <40% (3). The most important factor in the prognostic evaluation of HNSCC is the pathological staging (tumor-node-metastasis); in particular, the nodal stage ($pN$). However, it is well known that patients with HNSCC in the same stage can have different prognosis. Therefore, current work focuses on the identification of molecular and biological prognostic factors, which contribute to further differentiation among these parameters, cellular oncogenes, tumor suppressor genes, cell cycle control genes, and mismatch repair genes are under investigation.

PLKs, named after the Drosophila gene product polo, were implicated in the regulation of various steps of the cell cycle, such as activation of the phosphatase CDC25, bipolar spindle assembly, and cytokinesis. Recent data suggest that the activity of the polo homologue Plx1 in Xenopus laevis is required for the Ca-induced transition of M phase-arrested extracts to interphase, and they demonstrate an important role of Plx1 in the activation of the proteolytic machinery that controls exit from mitosis. In addition, proteolysis, which is also controlled by the anaphase-promoting complex, has a crucial role in controlling the passage of cells through anaphase: In Saccharomyces cerevisiae, the CDC5 gene encodes a protein kinase Cdc5p of the polo family, which participates in switching on proteolysis of mitotic cyclins. (6) It could also be demonstrated that Cdc5p is an unstable protein, the degradation of which is regulated by anaphase-promoting complex. These recent observations underline the central role of PLK for the progression of eukaryotic cells through mitosis. Our investigations of human PLK in various cell lines, as well as primary cells (activated lymphocytes and tumors of various origin), revealed that the mRNA and protein expression of PLK correlates with the mitotic activity of cells, suggesting PLK to be a novel marker for cellular proliferation (7–9).

In the present study, we examined 89 patients with HNSCC and found elevated PLK mRNA expression in the vast majority of primary tumors. $pN$ stage, as the most crucial prognostic factor for HNSCC patients, related also to PLK expression. On the basis of an observation period of 5 years after therapy, we demonstrated that determination of the PLK mRNA levels is of prognostic value for the patient population.

**Materials and Methods**

**Patients and Tumor Samples.** The study group consisted of 89 patients with HNSCC who were treated at the department of otorhinolaryngology of the J. W. Goethe-University, School of Medicine (Frankfurt, Germany). The cohort encompassed 78 male and 11 female patients who were smokers and ingested alcohol on a regular basis. Their ages ranged from 22–84 years (median, 54 years). Sixty-six patients suffered from oropharyngeal carcinomas, and 23 patients suffered from larynx carcinomas. In all cases, vital tumor probes from the primary site and microscopically normal tissue 5 cm away from the tumor margins were removed during surgery, immediately frozen in liquid nitrogen, and stored at $-80^\circ$C. Pretreatment staging of patients involved endoscopy of the upper aerodigestive tract, esophagoscopy, and bronchoscopy. Furthermore, computed tomography scan of the head and neck, chest X-ray, and sonography of the neck and abdomen were performed. All 89 patients underwent complete resection of the primary tumor and unilateral elective neck dissection for the clinically negative neck. In the case of clinically positive lymph nodes, unilateral or bilateral neck dissection was performed, depending on the involvement of lymph nodes. All neck dissections were carried out as modified radical neck dissections type III (10). According to histological criteria, all tumors were classified as SCCs, WHO grading I–III.

The postsurgical stage of each patient was grouped according to the tumor-node-metastasis system. The majority of patients were stage IV ($T_{3+}N_{M0}$, $n = 36$; $T_{3+}N_{M0}$, $n = 19$), the remainder were stage III ($T_{3+}N_{M0}$, $n = 9$), stage II ($T_{3}N_{M0}$, $n = 15$), and stage I ($T_{1}N_{M0}$, $n = 10$). Whereas 25 patients (stages I and II) underwent surgery alone, 64 patients (stages III and IV) were subjected to surgery and postoperative radiotherapy.

Standard radiation schedule was used with 2 Gy fractions given as once-a-day treatment to a total dose of 70 Gy applied to the primary region and the neck. Chemotherapy was given nonconcomitantly, applying three cycles of cisplatin (20 mg/m$^2$/day) and 5-fluorouracil (1000 mg/m$^2$/day), according to a standard schedule (11). Tumor recurrences were treated chemotherapeutically with the same regimen until no response or progressive disease was measured in two dimensions by computed tomography scan. Follow-up information was obtained from posttherapeutically standardized routine investigations over a...
period of 5 years. Forty-nine patients suffered from tumor recurrence developing mostly regional metastases. Only three patients developed distant metastases (lung). The median time to recurrence ranged between 2 and 61 months after treatment (median, 8.5 months). Median survival time of deceased patients was 15.5 months, with a range from 1–80 months. The median quality of life index during the observation period with reference to the Eastern Cooperative Oncology Group scale was 3.

RNA Isolation and Northern Blots. Storage of tissues, RNA isolation, labeling of probes (PLK-17 low, GAPDH-low), Northern blots, and hybridizations were performed as described previously (7). The uniformity of RNA loading was controlled by ethidium bromide-staining of the agarose gels. The membranes were stripped and rehybridized with a probe for GAPDH under identical conditions to normalize the quantity and quality of the agarose gels. For a comparison of PLK mRNA in HNSCC, RNA was isolated from normal and corresponding unaffected tissues, a Wilcoxon test was performed. Further, values of the rectangle for the area integration as peak value above background. In all cases, the entire lane was monitored to insure objective comparisons by entire lane from the gel pocket to the bottom edge of the original gel. In all cases, the entire lane was monitored to insure objective comparisons by

Statistical Analysis. For a comparison of PLK expression in tumors and corresponding unaffected tissues, a Wilcoxon test was performed. Furthermore, a Kruskal-Wallis test was applied to compare the PLK expression in various tumor stages. The relationship between PLK expression and survival time was analyzed according to the Kaplan-Meier method, using the log rank statistics. The survival statistic was based on the actuarial adjusted (disease-specific) survival rate. No patient was excluded from the survival analysis. Multivariate analyses were performed with the Cox’s proportional hazards model. Due to the exploratory layout of the study, no adjustment of type one error was given, and, therefore, all P's are interpreted per comparison. A statistical analysis was undertaken with the software package SPSS for Windows (4.0).

Results

PLK mRNA Is Overexpressed in Human Head and Neck Tumors. In previous studies, we have demonstrated that the expression of PLK mRNA and PLK protein correlates to the proliferative activity of cells (7–9). Although most adult tissues containing a low percentage of dividing cells exhibited PLK mRNA levels that were at the limit of detection in Northern blot experiments, enhanced levels were frequently found in human tumors. In contrast to all HNSCC samples (n = 89), which exhibited a prominent signal of 2.3 kb representing PLK transcripts in normal mucosa, only low levels of PLK mRNA were detected (Fig. 1).

For each patient, the ratio of PLK and GAPDH signals was determined. In microscopically normal mucosa, we found a median value of 0.22 d.u. for the normalized PLK expression ranging from 0.12–0.48 d.u. In cancer samples derived from oropharynx and larynx, levels of PLK mRNA were elevated and ranged up to 13.2 d.u. (mean, 1.13 d.u.; median, 0.83 d.u.; Fig. 2). Comparing unaffected tissues to corresponding tumors, we found a 4-fold (median) elevation of PLK mRNA expression in SCCs.

Overexpression of PLK mRNA Correlates to Prognostic Parameters and to the Survival of Patients with Head and Neck Cancer. To determine whether the PLK status was associated with the outcome of tumor patients, we analyzed the correlation of PLK expression to the pN stage and the disease-related survival. pN stage, as the most important prognosis factor in head and neck cancer, correlated to the PLK mRNA level with an overall significance of P = 0.0043 (Kruskal-Wallis). However, we could detect significant differences only between pN0 and pN1 (P = 0.0274), pN0 and pN2 (P = 0.0045), as well as pN0 and pN3 (P = 0.004). Differences between stages pN1, pN2, and pN3 did not reach the significance level of 95%.

According to a Kaplan-Meier analysis with median cutoff (PLK expression = 0.83 d.u.), patients exhibiting PLK expression <0.83 d.u. had longer survival times than those with tumors expressing ≥0.83 d.u. The median survival times were 53 months (95% CI, 35–70) and 25 months (95% CI, 16–33), respectively (log rank, P = 0.0246; Fig. 3A). To define a threshold of normalized PLK expression that provided maximal statistical significance in Kaplan-Meier tests, we performed the analysis in steps of 0.1 d.u. We could determine maximal significance (P = 0.0047) if the threshold was 0.5 d.u. When patients were grouped according to this threshold of PLK expression, we found a median survival time of 25 months (95% CI, 16–33) in the group (n = 57) with high PLK expression (≥0.5 d.u.) versus a median survival time of 63 months (95% CI, 42–83) in the group (n = 32) with moderate PLK expression (<0.5 d.u.; log rank, P = 0.0047; Fig. 3B).

A pN-based survival analysis showed that pN0- and pN1-staged patients had a significant better survival than patients staged pN2 and pN3 (P = 0.0045; Fig. 4A). The median survival times were 30

![Fig. 1. Expression of PLK mRNA in HNSCC. RNA was isolated from normal and malignant tissues of head and neck cancer. Samples (30 µg) of total RNA were fractionated by electrophoresis through a 2.2 M formaldehyde/1% agarose gel. Hybridization was done under high stringency conditions with antisense probes for PLK (top) and GAPDH (bottom). T, RNA isolated from tumor tissues; N, RNA derived from normal mucosa; A-D, RNAs derived from the same patient.](image)

![Fig. 2. Histogram of PLK mRNA levels. PLK mRNA expression in SCC of the oropharynx and larynx (n = 89). Specification in d.u.: mean = 1.12; median = 0.83; minimum = 0.04; maximum = 13.2; skewness = 4.04.](image)
months for patients staged pN 0/1 (95% CI, 24–35) and 63 months for
technology in stages pN 2/3 (95% CI, 25–100), respectively. Survival

differences between pN 0 and pN 1 , as well as between pN 2 and pN 3 ,
did not reach a statistical significance. For comparative purposes, we
combined pN stage and $PLK$ expression based on a cutoff point of
0.5 d.u. $PLK$ expression levels ($\leq 0.5$; $> 0.5$) discriminated the pN 0/1
group ($P = 0.0176$), as well as the pN 2/3 group ($P = 0.0061$), in a
prognostic better and worse population (Fig. 4, B and C).

To further test this selectivity of $PLK$ prognostic information, we
performed a multivariate analysis based on Cox regression analysis.
We used the backward selection method with the likelihood-ratio
statistic based on conditional parameter estimates. Age, pT stage, pN
stage, and $PLK$ mRNA expression, as well as sex, tumor site, and
therapy, (categorical variables) were subjected to the regression
model. Null hypothesis that all parameters (variables) of this model
are 0 was rejected by the score statistic (global $\chi^2$) and the likelihood
statistic with $P < 0.01$. From the variables entered in the full model,
only pN and $PLK$ reached significance with a hazard rate $e^{B_1} = 1.76$
(95% CI, 1.17–2.64) for pN ($P = 0.0063$) and a hazard rate $e^{B_2} = 1.18$
(95% CI, 1.06–1.41) for $PLK$ ($P = 0.0421$). During stepwise back-
ward selection, variables age, sex, pT, tumor site, and therapy were
eliminated so that only covariates pN and $PLK$ were in the equation of
the final model (score statistic, likelihood statistic: $P < 0.01$). The
hazard rate ($e^{B_1}$) in the final model was $e^{B_1} = 1.70$ (95% CI, 1.13–2.55)
for pN ($P = 0.0097$) and $e^{B_2} = 1.21$ (95% CI, 1.03–1.46) for $PLK$
($P = 0.0198$). None of the two variables was eligible for removal
because the observed significance level was <0.01 for all of them
(conditional likelihood estimates).

**Discussion**

Prognosis of patients with head and neck cancer today is based
mainly on clinicopathological parameters, especially on tumor site

![Kaplan-Meier curve of survival (n = 89) in relation to PLK expression (median cutoff): comparing patients with tumors exhibiting PLK expression $<0.83$ d.u. (median cutoff) with those with tumors expressing PLK at elevated levels ($\geq 0.83$ d.u.). Significant differences between both groups were found ($P = 0.0246$). B, Kaplan-Meier curve of survival (n = 89) in relation to PLK expression (cutoff = 0.5 d.u.): comparing patients with tumors exhibiting PLK expression $<0.5$ d.u. (n = 32) with those with tumors expressing PLK $\geq 0.5$ d.u. (n = 57). Optimal significance was obtained when patients were grouped according to this threshold of PLK expression ($P = 0.0047$).](image)

![Kaplan-Meier curve of survival (n = 89) in relation to pN stage and PLK expression. A, pN stage: significant differences were only found between pN 0/1 and pN 2/3 ($P = 0.0045$). B, patients staged pN 0/1 (n = 34) can be subdivided into groups with different outcomes according to PLK grading ($P = 0.0176$). C, patients staged pN 2/3 (n = 55) can also be subdivided into different prognostic groups based on a PLK threshold of 0.5 d.u. ($P = 0.0061$). PLK expression $<0.5$ d.u. (n = 18); PLK expression $\geq 0.5$ d.u. (n = 36).](image)
and regional lymph node involvement. However, it is generally accepted that tumors of the same stage can behave differently, leading to variable results concerning tumor-free survival after therapy, as well as overall survival. In recent years, a growing spectrum of biochemical investigations has contributed to better characterize biological tumor behavior, thereby improving treatment strategies. Among these studies, we overlook a series of investigations in the field of head and neck cancer concerning the expression of p53, the epidermal growth factor receptors and certain ligands (transforming growth factor α), as well as the proliferation-associated markers PCNA and Ki67 (12). Because Ki67 and PCNA can easily be studied using classical immunohistochemical techniques, in recent years, several investigators focused their attention to the clinical impact of these markers for cellular proliferation (13–17). Still, a controversial discussion is held on the potential of proliferation markers, such as Ki67 and PCNA, for the determination of the patient’s prognosis, dependent or independent of regional lymph node involvement and its impact on the therapy decision (18, 19). We have previously cloned human PLK belonging to an evolutionary conserved family of cell cycle regulators (7). PLK codes for a serine/threonine kinase, which is required for the maturation of mitotic spindles, involved in the progression of mitosis (4). Our investigations on human PLK with various cell lines, activated lymphocytes, and tumors of various origin showed that the expression of PLK correlates to the mitotic activity of cells (7–9). PLK transcripts are elevated in a variety of malignant tumors (7–9). In the present study, we examined 89 patients with HNSCC for PLK mRNA expression in their primary tumors. We found PLK mRNA to be overexpressed in the majority of SCCs of the oropharynx and larynx. Because PLK is mainly expressed in the transition of late G2-M phase of the cell cycle (4), its overexpression seems to reflect a higher percentage of cells in HNSCC being in the G2-M phase at a random point of investigation compared with normal cells. Moreover, PLK expression turned out to be significantly higher in metastatic HNSCC than in nonmetastatic tumors. This observation suggests that determination of PLK expression in primary tumors of the oropharynx and larynx is able to indicate the metastasizing potential of a tumor.

The metastasizing capability of cancer may depend on the proportion of proliferating cells, duration of the cell cycle and cell death, as well as the microenvironment (host factors) of the primary tumor. Therefore, PLK seems to determine the fraction of proliferating cancer cells, which enter further cell cycles and drive the tumor into clonal heterogeneity with cells that exhibit enhanced metastasizing behavior. Moreover, the fact that cancer patients in early stages (pN0), as well as in late stages (pN2/3), could be further subdivided by PLK expression in patients with better or worse prognosis, suggests PLK mRNA levels as “discrimination marker” within conventional tumor stages. The evaluation of significant variables for tumor progression and patients’ outcome by Cox’s proportional hazards model confirms this hypothesis. One unit (d.u.) increase of PLK results in an estimated increase of 20% in the patient’s risk of dying. PLK improves the prediction based on the pN stage by an additional contribution and is an independent significant predictor of survival. By using a combination of the two criteria “pN” and “level of PLK mRNA,” the aggressiveness of HNSCC and the patient’s risk of dying can, therefore, be judged more precisely, thereby improving the definition of a suitable therapy. For example in an early-stage, high PLK expression in the tumor could be a reason to favor an adjuvant therapy. On the other hand, late-stage therapy must not necessarily be performed in each case with the same aggressiveness. Our ongoing investigations have to prove these hypotheses also with more practicable immunohistochemical methods evaluating PLK as marker for routine diagnostics. Nevertheless, experiments to inhibit PLK expression in head and neck cancer cell lines are under current investigation in our laboratory. Preliminary results have shown that PLK-specific antisense oligonucleotides to PLK reduce the mitotic activity of proliferating cells.5 Thus, apart from its diagnostic value, PLK may be of therapeutic interest in SCC of the head and neck.

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References
