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Influence of neuroendocrine tumor cells on proliferation in prostatic carcinoma

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Summary Neuroendocrine (NE) tumor cells in prostatic carcinoma (PCa) may influence tumor proliferation by a paracrine stimulus. The role of NE tumor cells is discussed controversially. This study investigates the influence of NE tumor differentiation on proliferation in PCa. Neuroendocrine differentiation, Ki-67, and Polo-like kinase 1 were studied immunohistochemically in 73 consecutive prostatectomies. Polo-like kinase 1 (PLK1) expression was also studied by Western and Northern blot analysis. Tumors were classified as high NE (HNE) and low NE differentiated (LNE), and depending on the growth pattern, with solitary and clusters of NE tumor cells. Low NE differentiated tumors were defined as less than 30 and HNE as 30 or more NE tumor cells per hot spot. Patients were followed by serum prostate-specific antigen (PSA) analysis. Neuroendocrine differentiation was present at least focally in 70% of tumors; 57% were HNE and 43% LNE. Solitary NE tumor cells were more often found in low-grade PCa, whereas clusters of NE tumor cells were more frequent in high-grade PCa. PLK1 messenger RNA and protein as well as Ki-67 were overexpressed in tumor tissue compared with tumor-free tissue. A stronger proliferation as determined by Ki-67 and PLK1 expression was present in HNE tumors compared with LNE tumors and in tumors with clusters in contrast to tumors with solitary NE tumor cells. Analysis for PSA relapse-free survival showed an earlier progression in HNE than in LNE tumors and in PCa with clusters of NE tumor cells. A significant and clustered NE differentiation in PCa may lead to an increased proliferation and earlier tumor progression, whereas few and solitary NE tumor cells have no prognostic impact.

1. Introduction

Prostatic carcinoma (PCa) has evolved as the most frequent malignant tumor in males and is already second among cancer related deaths in men [1]. Neuroendocrine

Abbreviations: PLK, Polo-like kinase; PCa, Prostatic carcinoma; CgA, Chromogranin A; NE, Neuroendocrine; HNE, High neuroendocrine differentiation; LNE, Low neuroendocrine differentiation; PI, Proliferation index.

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(NE) differentiation of tumor cells can frequently be found in exocrine PCa to a various extent [2]. The origin of this tumor cell type is not yet clear, but there are hints that a shift from an exocrine to a NE phenotype occurs [3]. Neuroendocrine tumor cells lack the androgen receptor and are therefore androgen independent [4]. Upon androgen withdrawal the androgen-dependent cell line LnCaP can be transdifferentiated into an NE phenotype by increasing the cyclic adenosine monophosphate in vitro and in vivo [5]. This effect can also be observed in man, where an increased NE tumor differentiation is present in androgen-independent PCa [6]. Most frequently, NE tumor cells occur as solitary tumor cells within the exocrine tumor compartment. But as well as the solitary occurrence of these cells, small and large clusters of NE tumor cells can be found within tumor tissue [7]. Neuroendocrine tumor cells can secrete various peptide hormones such as bombesin, calcitonin, and parathyroid-related hormone that can mediate a proliferative stimulus to the microenvironment by a paracrine mechanism [3,8]. Therefore, an increased proliferation rate in the surrounding tumor tissue could be demonstrated [9-11]. Proliferation markers could not be detected in NE tumor cells so far. The NE tumor cell type is therefore regarded as postmitotic [12-14]. In previous studies we have demonstrated that NE tumor cells, when occurring in a considerable amount, have an influence on the microenvironment and can induce an increased neovascularization. In addition, the occurrence of different sizes of clusters of NE tumor cells was investigated [7]. In high-grade tumors, large clusters of NE tumor cells were observed more frequently than in low-grade tumors.

Polo-like kinase 1 (PLK1) is a proliferation-associated protein that is related to the protein polo of *Drosophila melanogaster* and Cdc5 of *Saccharomyces cerevisiae*. PLK1 is a member of the family of serine/threonine kinases and interacts with the spindle apparatus during mitosis [15]. PLK1 is an essential protein during the M phase of mitosis. Inhibition or mutation of PLK1 results in failure of formation of a mitotic bipolar spindle and cells produce only monopolar spindles [16]. Although PLK1 is M phase–specific, basal amounts of the protein are expressed during the whole cell cycle except the G_0 phase [17]. PLK1 therefore represents a new proliferation marker and studies in non-small cell lung carcinoma, head and neck squamous cell carcinoma, and malignant melanoma showed a correlation to the prognosis and stage of disease [18-20]. In PCa, Ki-67 could be demonstrated as an independent prognostic marker in earlier studies [21,22]. The expression of PLK1 in PCa has been demonstrated in a previous study with no significant correlation to patients' prognosis [23]. In this study we examined the PLK1 expression and the established proliferation marker Ki-67 to investigate the influence of the NE tumor differentiation on proliferation.

2. Materials and methods

2.1. Patients and tissue sampling

Seventy-three consecutive prostate glands removed by radical prostatectomy from patients with no prior therapy were sampled. The mean age of patients was 65 ± 6.3 years. Patients' informed consent was obtained before all investigations. Tumor-suspicious areas were resected and snap frozen in liquid nitrogen and stored at −80°C until use. The remaining material was fixed in 4% buffered formaldehyde for 24 hours and sectioned following standard protocols [24]. The frozen sections of the native material identified tumor and tumor-free areas and were included in the diagnostic process. The grading was performed in accordance to Gleason [25]. Low-grade tumors were defined as tumors with Gleason scores 2 to 6. Subgroup analysis of tumors with Gleason scores 7 to 10 with respect to NE tumor differentiation, proliferation index (PI), and PLK expression revealed no difference between tumors with Gleason scores 7 and 8 to 10 (data not shown); therefore, high-grade tumors were defined as Gleason scores 7 to 10. Tumor staging was performed according to the sixth edition of the International Union Against Cancer TNM classification (2002). An organ-confined stage (pT2a/2b/2c) was found in 31 cases and an advanced stage (pT3a/3b; pT4) in 42 cases. In 3 cases, lymph node metastases were present. Follow-up data were available in 70 patients. The mean follow-up time was 20.7 ± 12.0 months (median, 21 months; range, 3-44 months). Patients were followed by screening of the prostate-specific antigen (PSA) serum levels. A postoperative increase in PSA of more than 0.1 ng/mL was considered as tumor recurrence/progression.

2.2. Immunohistochemistry and double staining

The following antibodies and dilutions were used: chromogranin A (CgA, monoclonal, clone LK2H10) ready to use (Linaris, Wertheim-Bettingen, Germany); PLK1 (monoclonal, clone PLK1) 1:200 (BD Biosciences, Heidelberg, Germany); Ki-67 (monoclonal, clone MIB-1) 1:20 (Dianova, Hamburg, Germany). The incubation with primary antibodies for CgA and Ki-67 was performed for 1 hour at room temperature whereas for PLK1 it was 2 hours at room temperature in a dark humid incubation chamber. Antigen retrieval for Ki-67 was performed in a microwave oven 3 times for 5 minutes and 1 time for 5 minutes followed by 8 times for 3 minutes for PLK1 in citrate buffer (pH = 6.0). For CgA, unmasking procedures were not necessary.

Serial sections (3 μm thick) were cut in a SM200R microtome (Leica, Bensheim, Germany) and mounted on silane-coated glass slides. Sections were dried in an incubation chamber at 37°C and deparaffinized according to standard protocols. Visualization of antigenic sites was performed using the Envision staining kit (DAKO, Hamburg, Germany) using 3-amino-9-ethylcarbazol. Double