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DISSERTATION

Core classification, DNA ploidy and HPV in lung and head and neck cancer

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Zusammenfassung

Lungen und Kopf-Hals-Karzinome haben ähnliche genotoxische Riskofaktoren. Während die Mehrzahl der Lungenkarzinome allein durch das Zigarettenrauchen verursacht wird, ist das Rauchen zusammen mit einem Alkoholabusus die Hauptursache der Kopf-Hals-Karzinome. Humane Papilloma Viren (HPV) wurden identifiziert als wichtiger ursächlicher Faktor für Tonsillenkarzinome. Demgegenüber ist die chromosomale Instabilität und Aneuploidie, die durch eine DNA Messung nachgewiesen werden kann, vorrangig mit der Tumorprogression assoziiert.

Das Ziel unserer Studien war ein besseres Verständnis der Biologie und Pathologie der Lungen- und Kopf-Hals-Karzinome. Speziell sollte versucht werden a) eine mikroskopische Tumorklassifikation zu entwickeln, die Einblicke in die Genetik der Krebszellen und insbesondere deren DNA Ploidie erlaubt, b) diese Klassifikation auf Lungen- und Kopf-Hals-Klassifikation anzuwenden und mit klinisch-pathologischen Parametern wie auch Biomarkern zu korrelieren, c) molekulare Charakteristika und insbesondere den HPV Status von Lungen- und Kopf-Hals-Karzinomen zu analysieren. Dafür wurde eine Kern-Klassifikation entwickelt, die auf der semiquantiativen Analyse der Größe und Art von Tumorzellkernen und -mitosen basierte. Es wurde herausgefunden, dass 1) die Kern- und Mitosegröße mit dem DNA Gehalt der Tumorzellen korrelierte, 2) tripolare Mitosen ein Indikator für nahe-triploide Karzinome sind, 3) morphologische und DNA Parameter, die auf eine Variabilität des Krebsgenoms hinweisen, mit einer schlechten Prognose von Lungenkrebspatienten assoziiert sind, 4) HPV positive Kopf-Hals-Karzinome durch kleinere Kerne und einen geringeren DNA als HPV-negative charakterisiert und 5) HPV mit dem Lungenkrebs in bestimmten geographischen Regionen der Erde verknüpft ist.

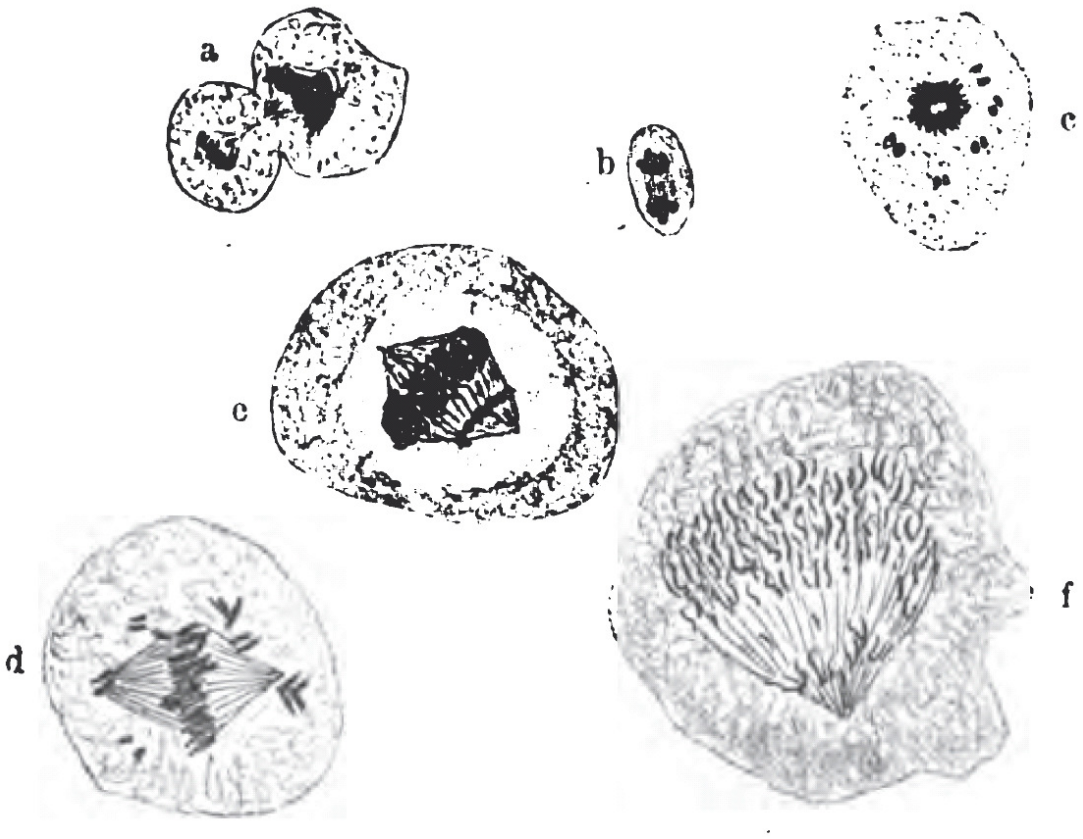
Summary

Lung, head and neck cancers have similar genotoxic risk factors. While the vast majority of lung cancer is caused by cigarette smoking alone, smoke together with heavy drinking are the major etiological agents of head and neck cancer. In addition, human papilloma virus (HPV) has been identified as an important causative factor of tonsillar carcinomas. In contrast, chromosomal instability and aneuploidy being identifiable by DNA measurement are predominant associated with cancer progression.

The aim of our studies was a better understanding of the biology and pathology of lung and head neck cancer. In particular, it was attempted a) to develop a microscopy-based tumor classification system that provides insight into the genetics of cancer cells and in particular their DNA ploidy, b) to apply this classification system to lung and head and neck cancer and to correlate it with clinicopathological parameters and molecular biomarkers and c) to analyze molecular characteristics and in particular the presence of human papilloma virus in lung and head and neck cancer. Therefore we developed a core classification which was based on the semi-quantitative assessment of the size and type of tumor cell nuclei and mitoses. It was found that 1) nucleus and mitosis size correlated with the DNA content of the tumor cells, 2) tripolar mitoses were indicative for cancer with near-triploid DNA content, 3) morphological and DNA parameters indicating variability of the cancer genome were associated with poor prognosis of lung cancer patients, 4) HPV-positive head and neck squamous cell carcinomas were characterized by smaller tumor nuclei and reduced DNA amount compared to HPV-negative carcinomas and 5) HPV is associated with lung cancer in certain geographical regions of the world.

Introduction

Lung, head and neck cancers have a similar genotoxic risk factor. While the vast majority of lung cancer is caused by cigarette smoking alone (Goeckenjan et al 2011) smoke together with heavy drinking are the major etiological agents of head and neck cancer. By the field cancerization patients have a high risk for the development of second primary tumours of the aerodigestive tract (Slaughter et al. 1953). Human papilloma virus (HPV) has been shown to be the causative agent for the majority of cervical cancers and has been meanwhile implicated in a wide variety of other cancer types (Petersen & Klein 2008).



Asymmetrie distribution of cell divisions and scattered mitosis (from Hansemann 1897)

In contrast, chromosomal instability and aneuploidy are known to play a role in cancer progression (Schulze & Petersen 2011). It may also be implicated in early tumor development as was already pointed out by Boveri about 100 years ago (Ried 2009). Asymmetric cell division and abnormal mitoses have even been described a couple of years earlier by Hansemann (Hansemann 1897, Fig. 1). Meanwhile we know that these changes are linked to chromosomal changes and genetic instability.

Despite these facts there has been to our knowledge no systematic analysis of morphological criteria like nucleus and mitosis size, their size variability or the presence or absence of tripolar or tetrapolar mitosis with DNA parameters that are able to identify aneuploidy or other parameters that can be linked with genomic aberrations.

Objectives

The overall aim was the attempt to gain a better understanding of the biology and pathology of lung, head and neck cancer. Specifically the following objectives were addressed:

1. Development of a microscopy-based tumor classification system that provides insight into the genetics of cancer cells and in particular their DNA ploidy.

2. Application of this classification system in lung, head and neck cancer and its correlation with clinicopathological parameters and molecular biomarkers.

3. Analysis of molecular characteristics and in particular the presence of human papilloma virus in lung, head and neck cancer.

Materials and Methods

Core classification of cancer

The rationale for the development of the core classification resided in the idea that current morphological classification did not specify the size of the tumor nuclei. The core classification is based on histomorphological criteria for scoring the nuclear size and the mitosis size (Fig. 2, see also table 1 in Petersen et al. 2009 and Kotb et al. 2010). In addition, the variability of these parameters is documented. Furthermore, it differentiates whether the following parameters are absent (score 0), present (score 1) or abundant (score 2): tripolar mitosis, tetrapolar mitosis, typical mitosis, atypical mitosis.

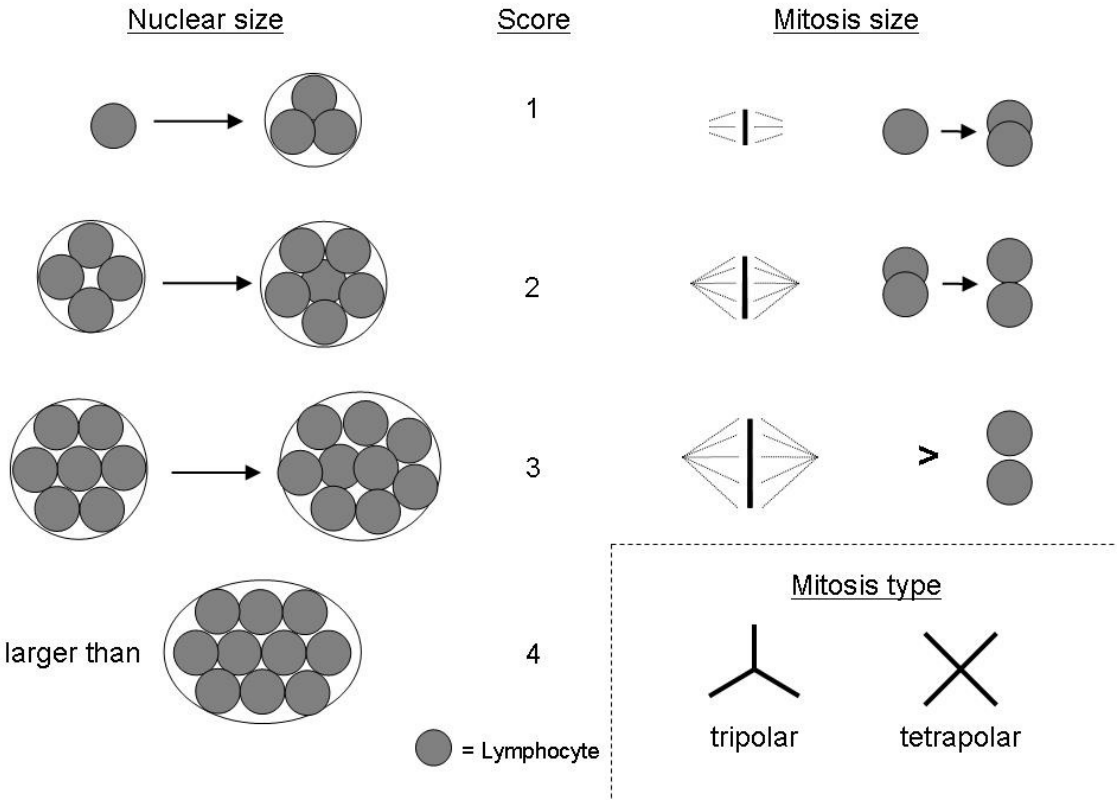


Fig. 2 Core classification of cancer. The tumor cell nuclei and mitoses are scored according to their size in relation to lymphocytes

DNA ploidy, HPV typing and biomarker analysis

DNA measurement was performed on Feulgen stained single cell suspensions by DNA image cytometry as described (Petersen et al. 2009, Kotb et al. 2010). Analysis for HPV was based on a PCR amplification followed by a chip hybridization for the differentiation of the most common HPV subtypes (Petersen et al. 2007, Kotb et al. 2010). The other biomarker analysis was done as described (Chen et al. 2007).

Statistical analysis

The statistical analysis involved correlation of the parameters of the core classification, the DNA ploidy measurement and the HPV typing with clinicopathological parameters and in particular survival data. For the latter, the Kaplan Meier was performed. Other statistical tests were applied as specified in the publications (Petersen et al. 2009, Kotb et al. 2010, Chen et al. 2007) and were done by using the SPSS software.

Literature searches and review

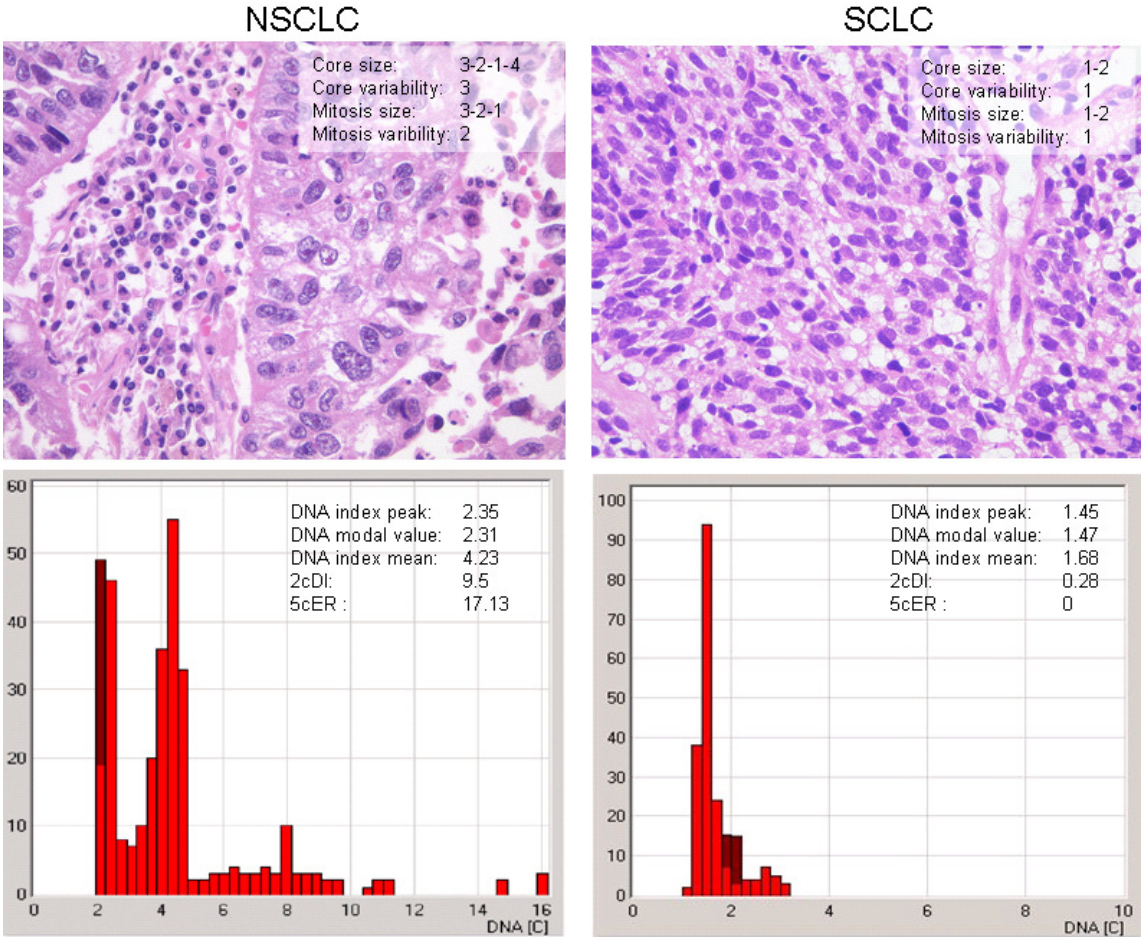
The analysis of the presence of HPV in lung cancer was based on published data using PCR as detection method. The publications were retrieved from the Pubmed database by search parameters as specified (Klein et al. 2009). All publications were analyzed according to frequency of HPV in the respective tumor collectives, the specific HPV types and their presence in distinct histological lung cancer subtypes. Furthermore, all data was represented according to the continent, country and region in which the studies were performed.

Results

Core classification and DNA ploidy in lung cancer

The main findings of the core classification and DNA ploidy analysis of lung cancer (Petersen et al. 2009) can be summarized as follows:

1) The nuclear size as well as the mitosis size scored according to the core classification in a semi-quantitative manner reflects the DNA content of tumor cells, i.e. larger nuclei indicate a hyperdiploid tumor being observed in non-small cell lung cancer while small nuclei may be associated with hypodiploidy as is typically seen in small cell lung cancer (Fig. 3).



Fig, 3 Increased nuclear/core size reflects the higher DNA content of non-small cell lung cancer (NSCLC) compared to small cell lung cancer (SCLC)

2) Tripolar mitoses are indicative of cancer with a near triploid DNA content (Fig. 4)

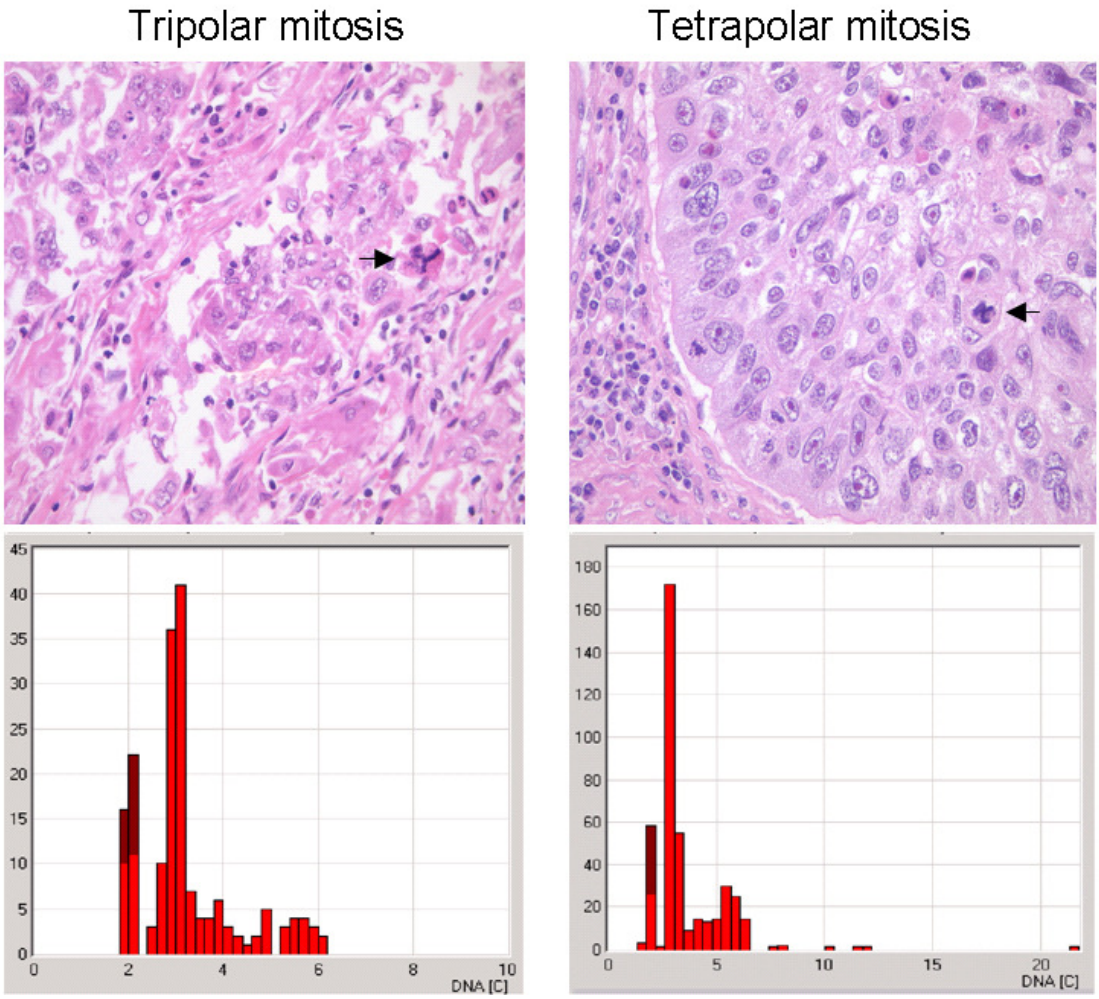


Fig. 4 Tripolar mitoses were correlated with a near triploid DNA stem line while tetrapolar mitosis were correlated with an increased DNA content but not with near-tetraploidy

3) Morphological and DNA parameters indicating increased variability of the cancer genome are associated with poor patient survival (Fig. 5)

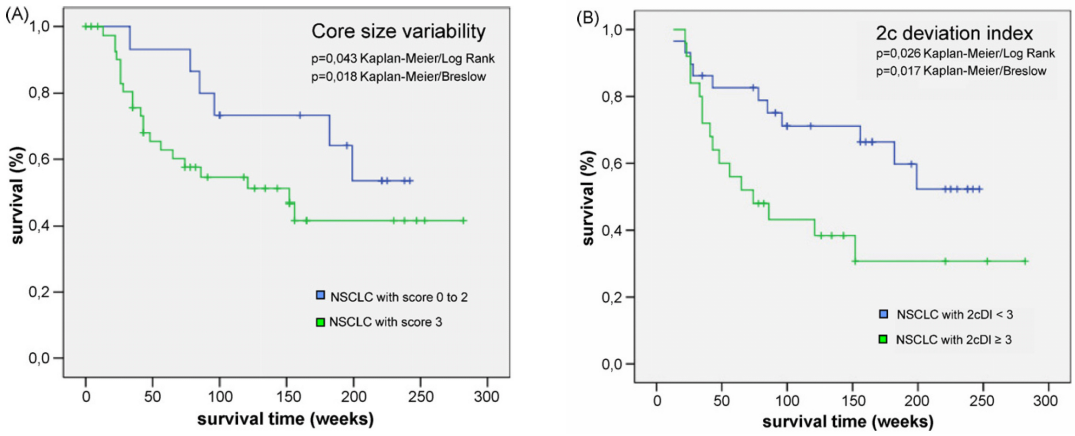


Fig. 5 Core size variability and 2c deviation index, parameters being indicative of genome instability, are associated with NSCLC patient survival (Petersen et al. 2009)

Core classification, DNA ploidy and HPV in head and neck cancer

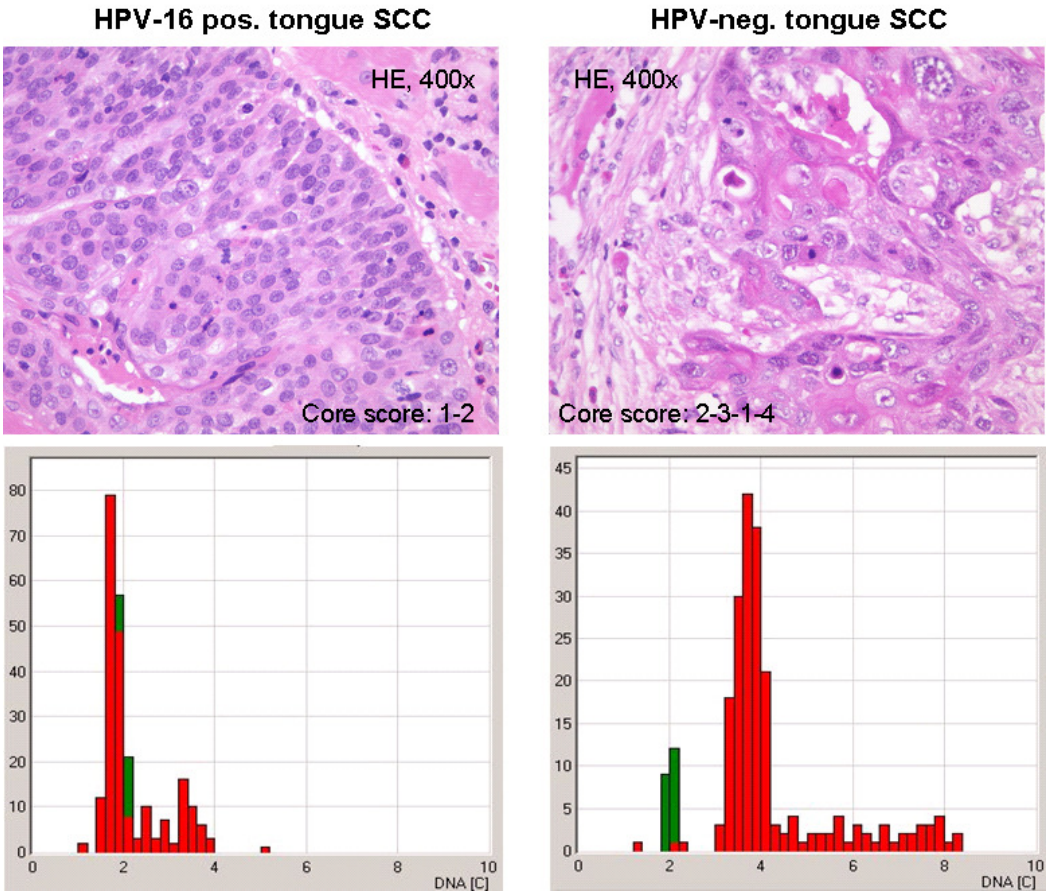


Fig. 6 Core classification, DNA ploidy and HPV analysis in HNSCC (see Kotb et al. 2010)

The application of the core classification of head and neck squamous cell carcinomas (HNSCC) confirmed the finding in lung cancer that increasing values of the nuclear size and mitosis size were significantly associated with higher indices of the DNA cytometry analysis. In addition, the analysis revealed that HPV-positive HNSCC had significantly smaller nuclei than HPV-negative cases (Fig. 6).

HPV and IGFBP7 in lung cancer

In the literature review on the incidence of HPV in lung cancer there was a considerable heterogeneity between certain countries and regions. Particularly high frequencies of up to 80% were seen in Okinawa (Japan) and Taichung (Taiwan). Overall, the mean incidence of HPV in lung cancer was 24.5%. While in Europe and America the average reported frequencies were 17% and 15%, respectively, the mean number of HPV in Asian lung cancer samples was 35.7%. However, there were also discrepant results within the same region pointing to methodological differences and the need for validation. All lung cancer subtypes were affected and especially the high risk types 16, 18, 31 and 33 as well as the low risk types 6 and 11 were found, the latter mainly in association with squamous cell carcinomas. The incidence of HPV, the virus subtypes and the tumor histologies are listed in Table 1 of the publication (Klein et al. 2009). Overall, the data suggested that HPV is the second most important cause of lung cancer after cigarette smoking and strongly argues for additional research on this issue.

We could show that the insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1), also termed IGFBP-7, is downregulated in human lung cancer due to methylation and that the gene may act as a tumor suppressor gene (Chen et al. 2007).

Discussion

Implication of the core classification in tumor analysis

Specifications of cell size have became part of the definition of many tumour entities and are particularly important for lung cancer. however They carry, several insufficiencies. The criteria for defining large cell lung carcinoma, for instance, are far less precise than for small cell lung cancer. As major inaccuracy, non of these descriptions differentiates between cell size and nuclear size which can differ considerably. Given the fact that the tumour behaviour is essentially governed by its genotype, we felt that there is a need to focus on the nuclear parameters of the tumour cell and therefore established the core classification. To our knowledge it was the first attempt to microscopically categorize tumours by the predominant size of their nuclei providing criteria for defining small, medium, large or giant core carcinomas. In addition, it represents a system to express the range of nuclear sizes of tumours and their relative frequencies and thus provides a measure for nuclear pleomorphism. The term “core” was chosen as it can be used as synonym for “nucleus”.

In addition, the classification seemed to have prognostic significance, since a high variability of the nuclear sizes was associated with poor patient survival. Meanwhile there have been additional attempts to categorize adenocarcinomas of the lung by the size of the tumor nuclei which resulted in a grading system with prognostic relevance (Nakazato et al. 2010) lending support to our approach.

A second important finding of our lung cancer study was the fact that tripolar mitoses were associated with a near triploid cancer stem line and thus a near-triploid DNA content of a major cancer cell subpopulation. In the meantime, our group performed a systematic analysis of the chromosome numbers in a multitude of tumor types (Schulze & Petersen 2011). The analysis of lung cancer is shown in Fig. 7 (Petersen et al. 2010). Interestingly, there was a considerable number of cases with a near triploid giving rise to a second peak in the corresponding histogram.

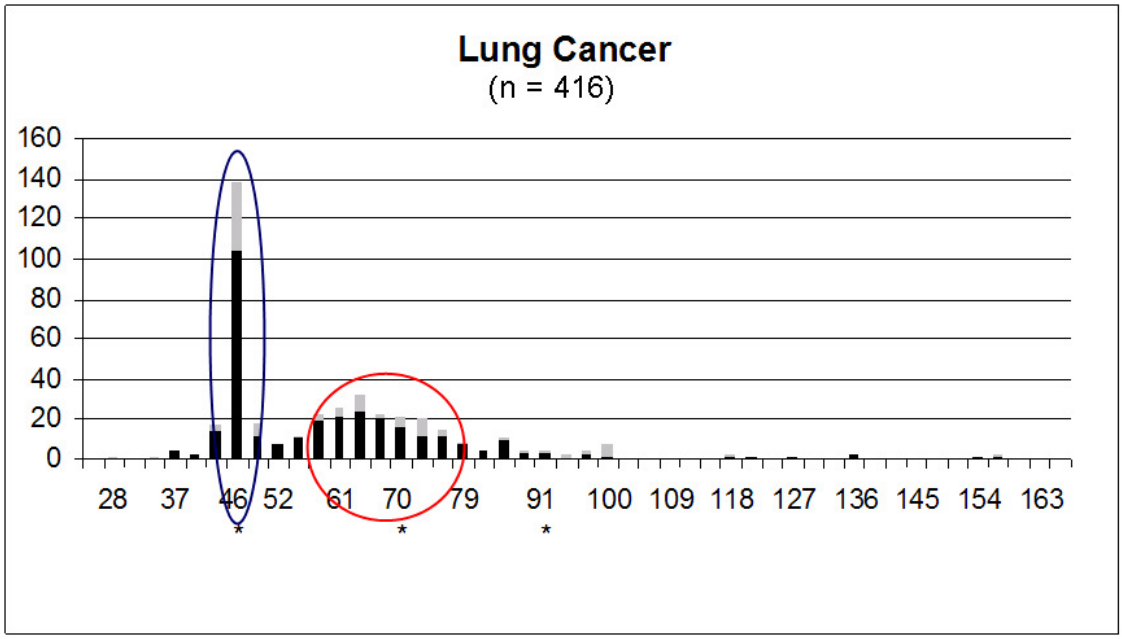


Fig. 7 Chromosome numbers of human lung cancer based on data from the Mitelman database for chromosome alterations. Apart from cases with a near diploid chromosome content there is a second population of lung samples with a near triploid chromosome content. The numbers of chromosomes are indicated at the x-axis while the number of cases may be deduced from the y-axis.

Lung cancer belonged to the group of tumor types with a considerable number of near triploid cancer samples. This phenomenon was also detected in other cancer types, particularly carcinomas. Thus the question arose whether these near triploid cancers may represent a biologically distinct cancer subgroup. In our analysis we could show that a high percentage of near-triploidy seems to be associated with poor patient survival (Fig. 8, Schulze & Petersen et al. 2011).

Meanwhile it was shown in stage I adenocarcinomas of the lung that atypical/tripolar mitosis is an indicator of worse prognosis (Kadota et al. 2011). Since tripolar mitosis is related to near-triploidy, this seem to support the observation that near-triploidy may have prognostic relevance. However, it should be mentioned that we could not detect a significant correlation between the presence of triploid mitosis and prognosis in our study (Petersen et al 2009). However, this may be due to the fact that our analysis of our tumor collective consisted

entirely of adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell cancer. Thus, the number of cases in the adenocarcinoma subgroup might have been too small to detect the association which was only found in stage I lung adenocarcinomas (Kadato et al. 2011).

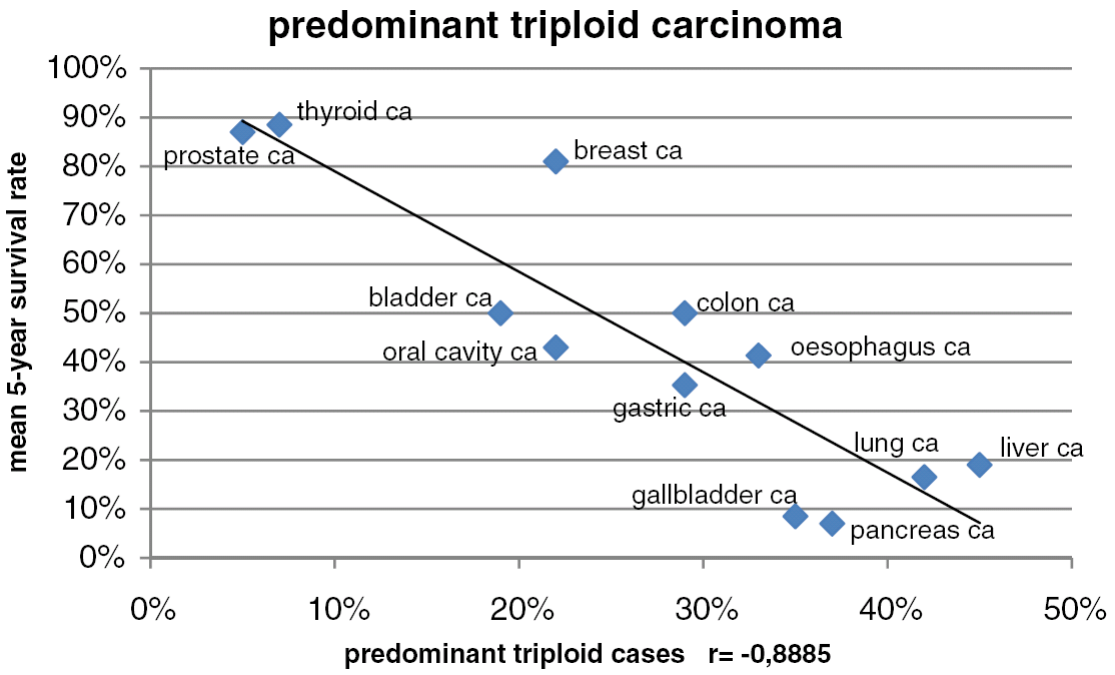


Fig. 8 Correlation between the percentage of predominant triploid carcinoma types and patient survival; The percentage of predominant triploid cases is shown at the X-axis, predominant triploid cancer samples were defined by chromosome number between 57 and 83. The mean 5-year survival of each cancer types is indicated at the y-axis. For details see Schulze & Petersen 2011.

Squamous cell carcinoma (SCC) of the lung carried the highest DNA content and largest tumor nuclei of all lung cancer subtypes (Petersen et al. 2009). Similarly, head and neck squamous cell carcinoma were frequently near tetraploid and showed large nuclei as well as tetrapolar mitoses (Kotb et al. 2010, Fig. 6). Interestingly, our study revealed a significant differences in HPV-positive and HPV-negative HNSCC suggesting that HPV carcinogenesis is less frequently linked to polyploidy than cancers caused by smoking and alcohol. It supported the fact that HPV positive HNSCC represents a tumor entity of its own, being

biologically and clinically different from non-HPV HNSCC (Klussmann et al. 2003, Fakhry, et al. 2008). Importantly, the core classification may be used to identify HNSCC with smaller tumor cell nuclei and potential HPV association.

HPV and other biomarkers in lung, head and neck cancer

HPV is known as the major risk factor for the development of cervical cancer. In addition, it has been associated with anal carcinomas and other cancer types of the genital region (Petersen & Klein 2008). In recent years, it has also become evident that it is a major cause of head and neck cancer (Will et al. 2006) and, in particular tonsillar carcinoma (Glombitza et al. 2010). In lung cancer, in contrast, HPV was long neglected as a potential cause of the disease although Syrjänen already suggested in 1979 that human papilloma virus could possibly be involved in bronchial squamous cell carcinoma (Syrjänen et al. 1979).

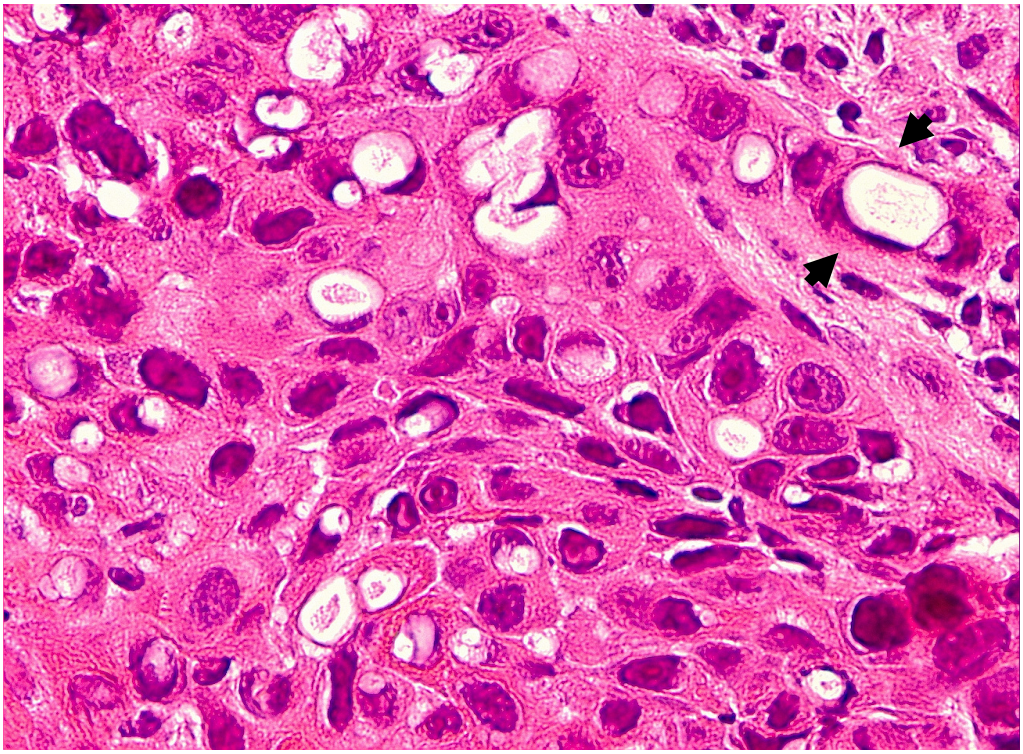


Fig. 9 Lung SCC with early invasion (arrowheads) that was positive for HPV-6 (Will et al. 2006)

After Syrjänen's first descriptions (Syrjänen et al. 1980, Syrjänen et al. 1987), several subsequent studies confirmed his findings (e.g. Stremlau et al. 1985, Tsuhako et al. 1998, Ciotto et al. 2006, Castelli et al. 2006, Will et al. 2006, Nadji et al. 2007). However, there have also been negative results (Welt et al. 1996) which might be due to the different detection methods or analyzed sample material (Giuliani et al. 2007a,b). We therefore decided to perform a systematic review of the literature and evaluated all data published on HPV in lung cancer by doing a Pubmed literature search (Klein et al. 2009). In Germany, lung SCC was only rarely associated with HPV. Only one German study could find HPV in 4% of the samples (Stremlau et al. 1985, Klein et al. 2009). Our group found low risk HPV only in an early invasive lung SCC (Will et al. 2006, Fig. 9). In contrast, there are regions particularly in Asia, in which HPV seems to be a major risk factor being detectable in up to 80% of cases (Hirayasu et al. 1996, Tsuhako et al. 1998, Miyagi et al. 2000). The interpretation of these results is difficult. However, it seems evident that HPV is associated with a distinct subset of lung cancer worldwide. The virus infection must be regarded as causative, at least it was shown that HPV was able to integrate into the tumor genome which is typical for HPV in cervical cancer, where it is an etiological agent (Aguayo et al. 2007). The exact incidence of HPV in lung cancer, however, still needs to be verified because there are obvious contradictions in the reported incidences even in the same geographical region (Papadopoulou et al. 1998, Gorgoulis et al. 1999).

HPV is a diagnostic biomarker that may point to the etiological cause of the disease. Since there is meanwhile a vaccine available, it is of clinical interest to identify all cancer types in which the vaccination may have a prophylactic benefit. IGFBP-7 can be regarded as a epigenetic biomarker since the gene does not seem to be inactivated by permanent mutations but rather DNA modifications that are potentially reversible. Apart from IGFBP-7, there are many other epigenetically silenced genes in lung cancer. Reactivation of IGFBP-7 may

constitute an interesting therapeutic target since the gene seem to have tumor suppressive properties (Chen et al. 2007). Our group is currently investigating other such genes that have a role in differentiation and may be reactivated by demethylating agents (Chen et al. 2011).

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Erklärung

Der Promovend Herr Waleed Farouk Mohamed Amin Kotb hat folgende Anteile an den vorgelegten Publikationen:

1. Chen Y, Pacyna-Gengelbach M, Ye F, Knösel T, Lund P, Deutschmann N, Schlüns K, **Kotb WF**, Sers C, Yasumoto H, Usui T, Petersen I. Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) has potential tumour-suppressive activity in human lung cancer. J Pathol. 2007 Mar;211(4):431-8.
10%

2. Klein F, **Amin Kotb WF**, Petersen I. Incidence of human papilloma virus in lung cancer. Lung Cancer. 2009 Jul;65(1):13-8.
30%

3. **Kotb WF***, Blind C*, Friedrich KH, Schewe C, Zhang ZG, Zheng JM, Deutschman N,Pacyna-Gengelbach M, Dietel M, Petersen I. Core classification of head and neck squamous cell carcinomas: correlations between morphology, DNA ploidy and HPV infection. Pathol Res Pract. 2010 Nov 15;206(11):768-71
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4. Petersen I*, **Kotb WF***, Friedrich KH, Schlüns K, Böcking A, Dietel M. Core classification of lung cancer: correlating nuclear size and mitoses with ploidy and clinicopathological parameters. Lung Cancer. 2009 Sep;65(3):312-8.
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Prof. Dr. Iver Petersen
Jena, 12.10.2011

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

Erklärung

„Ich, Waleed Farouk Mohamed Amin Kotb, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: **Core classification, DNA ploidy and HPV in lung and head and neck cancer** selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

Berlin den 06.08.2012 Waleed F. M. Amin Kotb