Putative Stem Cell Markers in Non–Small-Cell Lung Cancer
A Clinicopathologic Characterization

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Introduction: The cancer stem cell (CSC) theory postulates the existence of a distinct population of undifferentiated cells responsible for tumor initiation and maintenance. CSCs may be naturally resistant to the cytotoxic effect of radio-chemotherapy because of slow cell cycling, lower proliferation, and increased expression of DNA repair and antiapoptosis genes. To date, a universal marker for CSCs has not been identified. Proposed CSC markers are expressed both by cancer cells as well as by benign stem cells. Although many putative CSC markers exist, a precise characterization for non–small-cell lung cancer (NSCLC) is lacking.

Methods: We explored the expression of multiple alleged stemness associated markers in 371 surgically resected NSCLCs. Extensive clinical data and a postoperative follow-up period of up to 15 years enabled detailed clinicopathological correlations. ABCG5, ALDH1, CD24, CD44, CD133, CD166, epithelial cell adhesion molecule epitopes (ESA, MOC-31, Ber-EP4), nestin, OCT4, and sex-determining region Y-box 2 were analyzed immunohistochemically by using a standardized tissue microarray platform.

Results: Sex-determining region Y-box 2, CD44, ABCG5, ALDH1, and nestin were associated with poorer tumor differentiation and/or an increased proliferation index. ALDH1, CD44, and SOX2 were frequently found in squamous cell carcinoma, whereas CD24, CD166, and epithelial cell adhesion molecule markers were encountered in adenocarcinomas. CD44 expression was an independent marker associated with better overall survival in squamous cell carcinoma and Ber-EP4 was associated with tumor recurrences.

Conclusion: The expression and prognostic significance of CSC markers obviously varies depending on histologic NSCLC subtype. Importantly, our findings suggest that CD44 and Ber-EP4 may be promising for ongoing targeted therapies in specific NSCLC subgroups.

Key Words: Stem cells, Markers, Immunohistochemistry, Non–small-cell lung cancer.

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The cancer stem cell (CSC) theory postulates the existence of a distinct population of undifferentiated cells responsible for tumor initiation and maintenance.[1] The existence of CSCs was first proven in acute myeloid leukemia, and more recently in various other neoplasms including glioblastoma, melanoma, and epithelial cancers.[2–4] CSCs have the capacity for self-renewal, multipotency, and unlimited proliferation. CSCs may be naturally resistant to the cytotoxic effect of radio-chemotherapy because of slow cell cycling, lower proliferation, and increased expression of DNA repair and antiapoptosis genes.[5] These traits also characterize embryonic stem cells, thus suggesting probable overlap in the molecular signature between embryonic stem cells and CSCs.[6] Various alleged stem cell markers have been used for CSC identification and isolation, although detailed studies with phenotypic correlation are lacking. CD133 (prominin-1), a five-transmembrane glycoprotein, was initially described as a marker specific for human stem cells and their tumoral counterparts.[7] However, it is a matter of debate whether CD133 positive cells truly represent the ultimate tumorigenic population, apart from objective difficulties of staining specificity and interpretation.[8–10] The aldehyde dehydrogenase (ALDH) family represents cytosolic isoenzymes responsible for oxidizing intracellular aldehydes, contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation.[11] CSCs express metabolic enzymes such as ALDH1, which confer resistance to cyclophosphamide in normal stem cells.[5] Octamer binding transcription factor 4 (OCT4) belongs to the family of pituitary-specific positive transcription factor 1, Octamer transcription factor proteins 1 and 2, neural Unc (uncoordinated protein)-86 transcription factor from Caenorhabditis elegans (POU)-domain transcription factors involved in regulation of cell growth and differentiation. Its expression is normally confined to pluripotent cells of the developing embryo but OCT4 also plays a crucial role in maintaining CSC characteristics and resistive properties.[12] Another transcriptional factor, sex-determining region Y-box 2 (SOX2), also provides key signals for achieving characteristics of stem cells.[13] CD44, a cell-surface extracellular matrix receptor is commonly used as a CSC marker as well. CD44 can have multiple signaling functions (proliferation, apoptosis, survival, migration, differentiation), which depend on the cell type it is expressed on (embryonic, progenitor, cancer).[5] CD24 is a cell surface protein molecule functioning in cell–cell and cell–matrix interactions, thus playing a role in cell adhesion and metastasis.[14] Its role as a cancer stem cell marker

remains ambiguous, although CD24−/CD44+ tumor cells are often considered as CSCs, especially in breast cancer.[15] Nestin is known as a stem cell marker as well as a novel angiogenesis marker of neovascularization and epithelial cell adhesion molecule (EpCAM) has also been reported as part of the signature of cancer-propagating cells in numerous solid tumors and of normal progenitor and stem cells.[16,17] CD166, also known as activated leukocyte cell adhesion molecule, has been identified as a cell surface marker of certain hematopoietic progenitor cells as well as of pluripotent mesenchymal stem cells and is frequently considered a colorectal CSC marker.[18–20] The ABCG5 protein is a member of the ATP-binding cassette (ABC) subfamily G, which plays a role in the efflux transport of sterols and is thus involved in the development of resistance because of reduction of anticancer agents in cancer cells.[21,22] In colorectal cancer overexpression of EpCAM with ABCG5 has been observed and was associated with poor prognosis.[23] Furthermore, recent findings demonstrate that the two novel concepts, epithelial mesenchymal transition (EMT) and CSCs, have merged in cancer biology.[24] Studies show that EMT cells (e.g., epithelial cells with loss of E-cadherin expression) acquire stem cell characteristics, that maintenance of the stem cell state depends on continuous EMT-inducing signals, and that signaling pathways involved in stemness also act as potent EMT inducers.[24]

Proposed CSC markers show overlapping expression on other cancer cells and normal stem cells.[25] Although CSCs are typically present at very low levels, eradicating them could have high potential to more effectively treat tumors or remove residual tumor cells left after standard therapies, which bear a high risk for disease recurrence and metastasis. The identification and validation of CSC antigens for the generation of therapeutic antibodies may be a crucial factor, but is still in its infancy. Especially in lung cancer, one of the worldwide leading causes of cancer morbidity and mortality, CSCs have not been studied as intensively as in other cancers. Therefore, we aimed to explore the expression of multiple stemness associated factors in a well-documented cohort of surgically resected non–small-cell lung cancer (NSCLC) patients and to perform a correlative analysis with clincopathological parameters.[26]

MATERIALS AND METHODS

Patients and Tissue Sampling

The archival samples were derived from 371 NSCLC patients with radical surgical resection in curative intent between 1992 and 2004 and diagnosed at the Institute of Pathology, Medical University of Innsbruck.[27] Carcinoids were excluded from this analysis. Cases were selected only based on tissue preservation. Hematoxylin and eosin stained slides from all available specimens were initially reclassified by two pathologists (WS and AT) without knowledge of patient data, according to the current (2004) World Health Organization classification of tumors of the lung as described previously.[40] For the classification of adenocarcinomas (ACA) and distinguishing ACA from squamous cell carcinomas (SCC) and large cell carcinomas (LCC), the recently published multidisciplinary guidelines of the International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society were applied.[28] Tumor differentiation was graded as well, moderate, or poor. The clinical information was documented within the Twelve Years Retrospective of Lung Cancer survey, a project aiming to analyze various features of a large number of lung cancer patients.[29,30] Approval for data acquisition and analysis was obtained from the local Institutional Review Board, that is, the Ethics Committee of the Medical University of Innsbruck.

Tissue Microarray Construction

Tumor material consisted of paraffin-embedded tissue after fixation in 10% neutral buffered formalin. The tissue microarray (TMA) was constructed as previously described.[27] The first sections were stained by hematoxylin and eosin to confirm validity, the remaining sections were used for immunohistochemistry.

Immunohistochemistry

The primary antibodies and their applications are shown in Table 1. Staining protocols were followed according to the recommendation of the respective manufacturer. Immunohistochemistry was performed using the automated staining system Benchmark XT (Roche/Ventana Medical Systems, Tucson, AZ), except for ABCG5, CD133, CD166, and SOX2, which were incubated using the automated staining system Bond-maX (Leica Biosystems, Newcastle, United Kingdom). Positive control tissue for E-cadherin, Ber-EP4, epithelial-specific antigen (ESA), and MOC-31: skin and skin appendages; for SOX2 and OCT4: embryonal carcinoma; for CD24: Merkel cell carcinoma and an adenocarcinoma of the papilla Vateri; for nestin: gastrointestinal stromal tumor; for CD166: invasive ductal carcinoma of the breast; for CD44: diffuse large B-cell lymphoma; for ALDH1: ductal carcinoma of the pancreas; and for ABCG5: colon carcinoma.

Immunohistochemical Evaluation

Only spots containing at least 20 vital tumor cells were evaluated. If all four spots of a case did not meet this criterion it was excluded. Tumor cells were scored independently by two pathologists (WS, SS, EO, or AT) to study agreement between observers. The percentage of positively stained cells with clearly visible strong or moderate staining intensities, including staining localization was noted for each spot, followed by the calculation of the arithmetic mean value. The median percentage of positively staining tumor cells for each marker was chosen as the cutoff value for determining positive and negative cases, because the determination of cutoff values by receiver operating characteristic (ROC) analysis is less relevant in this setting.[31]

Statistical Analysis

The degree of agreement between observers was evaluated by interclass correlation coefficients, using reliability Cronbach's alpha analysis. Correlation analysis of clinicopathological and immunohistochemical parameters was performed using the Spearman test corrected for multiple testing,