Diagnostic and Prognostic Impact of Cytokeratin 18 Expression in Human Tumors: A Tissue Microarray Study on 11,952 Tumors

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Research article

Keywords: Cytokeratin18 (CK18), tissue microarray, immunohistochemistry

Posted Date: October 29th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-97788/v1

Version of Record: A version of this preprint was published on February 15th, 2021. See the published version at https://doi.org/10.1186/s10020-021-00274-7.

Abstract

Background

Cytokeratin 18 (CK18) is an intermediate filament protein of the cytokeratin acidic type I group and is primarily expressed in single-layered or “simple” epithelial tissues and carcinomas of different origin.

Methods

To systematically determine CK18 expression in normal and cancerous tissues, 11,952 tumor samples from 115 different tumor types and subtypes (including carcinomas, mesenchymal and biphasic tumors) as well as 608 samples of 76 different normal tissue types were analyzed by immunohistochemistry in a tissue microarray format.

Results

CK18 was expressed in normal epithelial cells of most organs but absent in normal squamous epithelium. At least an occasional weak CK18 positivity was seen in 90 of 115 (78.3%) tumor types. Widespread CK18 positivity was seen in 37 (31.9%) of tumor entities, including adenocarcinomas of the lung, prostate, colon and pancreas as well as ovarian cancer. Tumor categories with variable CK18 immunostaining included cancer types arising from CK18 positive precursor cells but show CK18 downregulation in a fraction of cases, tumor types arising from CK18 negative precursor cells occasionally exhibiting CK18 neo-expression, tumors derived from normal tissues with variable CK18 expression, and tumors with a mixed differentiation. CK18 downregulation was for example seen in renal cell cancers and breast cancers, whereas CK18 neo-expression was found in squamous cell carcinomas of various origins. Down-regulation of CK18 in invasive breast carcinomas of no special type and clear cell renal cell carcinomas (ccRCC) was related to adverse tumor features in both tumors ($p \leq 0.001$) and poor patient prognosis in ccRCC ($p=0.0088$). Up-regulation of CK18 in squamous cell carcinomas was linked to high grade and lymph node metastasis ($p<0.05$). In summary, CK18 is consistently expressed in various epithelial cancers, especially adenocarcinomas.

Conclusions

Down-regulation or loss of CK18 expression in cancers arising from CK18 positive tissues as well as CK18 neo-expression in cancers originating from CK18 negative tissues is linked to cancer progression and may reflect tumor dedifferentiation.

Introduction

Cytokeratin 18 (CK18) belongs to the cytokeratin acidic type I group (CK9-CK12) and is encoded by a gene located at chromosome 12q13 (1, 2). CK18 is an intermediate filament protein that forms heteropolymers with his co-expressed complementary type II keratin partner CK8, which assembles into keratin filaments - the major structural component in the cytoplasm of epithelial cells (1, 3). CK18 is

primarily expressed in single-layered or “simple” epithelial tissues of, for example, the liver, kidney, breast, prostate, gastrointestinal tract as well as in cancers arising from CK18 positive epithelial cells (4–7). Beside the important structural function, CK18 was also shown to play a role in apoptosis (8, 9), cell cycle progression (10), and cancer-related signaling pathways. For example, CK18 hypoglycosylation is linked to decreased Akt1 kinase activity and reduced cell survival (11). CK18 upregulation was described to be associated with decreased cell motility and invasiveness via the Wnt-pathway (12), and CK18 may be involved in the control of the ERK1/2-MAPK pathway (13, 14).

In surgical pathology, CK18 is used as an epithelial marker to identify CK18 positive adenocarcinomas that arise from different CK18 positive normal epithelia (4, 15). CK18 expression was also suggested as a potential prognostic marker. For example, decreased CK18 expression was found to be related to tumor progression in breast and colorectal cancers (16, 17). Elevated CK18 protein levels were found to be associated with unfavorable tumor features in oral and esophageal squamous cell carcinomas (18, 19) as well as in non-small cell lung cancers (20). CK18 antibodies have been used as diagnostic cancer markers for more than thirty years (4). However, the literature on the prevalence of CK18 expression is controversial for many cancers (21–47). For example, CK18 positivity has been described in 30% to 100% of oral squamous cell carcinomas (40, 47), 0–100% of non-small cell lung cancers (44, 46), and 0–43% of esophageal squamous cell carcinomas (18, 45). These conflicting data are likely to be caused by the use of different antibodies, immunostaining protocols, and criteria to determine CK18 positivity in these studies.

To better understand the prevalence and significance of CK18 expression in cancer, a comprehensive study analyzing a large number of neoplastic and non-neoplastic tissues under highly standardized conditions is needed. Therefore, CK18 expression was analyzed in more than 14,000 tumor tissue samples from 115 different tumor types and subtypes as well as 76 non-neoplastic tissue categories by immunohistochemistry (IHC) in a tissue microarray format in this study.

Materials And Methods

Tissue Microarrays (TMAs). Our normal tissue TMA was composed of 8 samples from 8 different donors for each of 76 different normal tissue types (608 samples on one slide). The cancer TMAs contained a total of 14,579 primary tumors from 115 tumor types and subtypes. Detailed histopathological data on grade, pT and pN status were available from 4,191 cancers (breast, kidney, bladder, various kinds of squamous cell carcinoma). Clinical follow up data were available from 1,178 breast cancer and 847 kidney cancer patients with a median follow-up time of 49/39 months (range 1–88/1-250). The composition of both normal and cancer TMAs is described in detail in the results section. All samples were from the archives of the Institutes of Pathology, University Hospital of Hamburg, Germany, the Institute of Pathology, Clinical Center Osnabrueck, Germany, and Department of Pathology, Academic Hospital Fuerth, Germany. Tissues were fixed in 4% buffered formalin and then embedded in paraffin. TMA tissue spot diameter was 0.6 mm. The use of archived remnants of diagnostic tissues for manufacturing of TMAs and their analysis for research purposes as well as patient data analysis has

been approved by local laws (HmbKHG, § 12) and by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

Immunohistochemistry. Freshly cut TMA sections were immunostained on one day and in one experiment. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at 121 °C in pH 7,8 buffer. Primary antibody specific for CK18 (mouse monoclonal, MSVA-118, MS Validated Antibodies, GmbH, Hamburg, Germany) was applied at 37 °C for 60 minutes at a dilution of 1:300. Bound antibody was then visualized using the EnVision Kit (Dako, Glostrup, Denmark) according to the manufacturer's directions. For tumor tissues, the percentage of positive neoplastic cells was estimated, and the staining intensity was semiquantitatively recorded (0, 1+, 2+, 3+). For statistical analyses, the staining results were categorized into four groups. Tumors without any staining were considered negative. Tumors with 1 + staining intensity in ≤ 70% of cells and 2 + intensity in ≤ 30% of cells were considered weakly positive. Tumors with 1 + staining intensity in > 70% of cells, 2 + intensity in 31–70%, or 3 + intensity in ≤ 30% were considered moderately positive. Tumors with 2 + intensity in > 70% or 3 + intensity in > 30% of cells werde considered strongly positive.

Statistics. Statistical calculations were performed with JMP 14 software (SAS Institute Inc., NC, USA). Contingency tables and the chi²-test were performed to search for associations between CK18 and tumor phenotype. Survival curves were calculated according to Kaplan-Meier. The Log-Rank test was applied to detect significant differences between groups.

Results

Technical issues. A total of 11,952 (82.0%) of 14,579 tumor samples were interpretable in our TMA analysis. The remaining 2,627 (18.0%) samples were not analyzable due to the lack of unequivocal tumor cells or loss of the tissue spot during the technical procedures. On the normal tissue TMA, a sufficient number of samples was always interpretable per tissue to determine the CK18 expression.

CK18 in normal tissues. CK18 was highly expressed in epithelial cells of the stomach (except parietal cells), duodenum, ileum, appendix, colon, rectum (Fig. 1A), gall bladder, pancreas (weaker staining in Islet cells than in acinus cells; Fig. 1B), endometrium, endocervix, alveolar cells of the lung, cytotrophoblast and syncytiotrophoblast of the placenta, and in all cells of the adenohypophysis (variable staining intensity). Liver tissue exhibited a zonal variability in hepatocyte staining ranging from negative to strongly positive (Fig. 1C). Bile ducts were always positive. Urothelium of the kidney and urinary bladder showed a strong staining in umbrella cells and a gradually decreasing staining intensity from superficial to basal urothelial cells (Fig. 1D). Salivary glands showed strong staining of serous and mucinous cells but somewhat weaker positivity in excretion ducts, especially in large ones. Some ducts only showed few positive cells or complete CK18 negativity. In the kidney, proximal and distal tubuli as well as collecting ducts were CK18 positive. In the ovary, follicular cells and follicular cysts stained positive as well as some cells of the corpus luteum. A strong positive staining of glandular cells with weaker and probably absent staining in basal cells was seen in prostate, respiratory mucosa of bronchus and paranasal sinuses,

epididymis, seminal vesicle, and breast glands (Fig. 1E). In lymph nodes, tonsil, spleen, and thymus delicate fibrillar staining caused by CK18 positive fibroblastic reticulum occurred mainly in the interfollicular area. In the thymus, some cellular components of Hassal bodies were CK18 positive, and merkel cells in the skin and hair follicles were CK18 positive. CK18 was absent in all mesenchymal tissues, the stroma of the ovary, posterior lobe of the pituitary gland, brain, bone marrow, lymph nodes, spleen and lymphocytes in tonsil and thymus. Staining was also negative in all squamous epithelia from esophagus, skin, lip, oral cavity (Fig. 1F), tonsil, and anal canal, hair follicles, sebaceous glands, testis (except some weak staining in some Sertoli cells in 2 of 8 samples), adrenal gland (except some cells with weak staining in 1/8 samples), aorta, heart, striated muscle, skeletal muscle, myometrium, muscular wall of the gastrointestinal tract, kidney pelvis, and the urinary bladder, corpus spongiosum of the penis, bone marrow.

CK18 in neoplastic tissues. Cytoplasmic immunostaining was observed in 9,098 (76.1%) of 11,952 analyzable tumors, including 45.0% with strong, 16.5% with moderate, and 14.6% with weak staining intensity. At least an occasional weak CK18 positivity was detected in 90 of 115 (78.3%) different tumor types and tumor subtypes and 78 (67.8%) tumor types and tumor subtypes had at least one tumor exhibiting strong positivity. Representative images of CK18 positive tumors are shown in Fig. 2. The highest frequencies of CK18 positivity were seen in adenocarcinomas of the lung, cervix uteri, small intestine, prostate, and pancreas, some breast cancer and thyroid cancer subtypes, and most of all neuroendocrine tumors and carcinomas. A detailed description of the immunostaining results is given in Table 1 and Fig. 3.

CK18 expression, tumor phenotype, and prognosis. The relationship between CK18 expression and clinico-pathological data could be analyzed in two cancer types derived from CK18 positive precursor cells (breast and kidney cancer), one cancer type derived from epithelium with variable CK18 expression (urinary bladder) as well as in 230 squamous cell carcinomas of various organs of origin (n = 8), but all derived from squamous epithelia that are normally CK18 negative (Table 2, Fig. 4). Reduced or absent CK18 immunostaining was associated with high UICC stage ($p = 0.0010$), high Thoenes grade ($p = 0.0086$), advanced tumor stage ($p < 0.0001$), and poor prognosis in clear cell renal cell cancers ($p = 0.0088$) and with high grade and unfavorable molecular features such as ER/PR negativity ($p < 0.0001$ each) - but not with patient outcome - in invasive breast carcinomas of no special type. In squamous cell carcinomas, CK18 up-regulation was preferentially seen in cancers with advanced stage (13.2%/136 pT1-2 vs 27.7%/94 pT3-4; $p = 0.0154$), presence of lymph node metastasis (14.7%/95 pN0 vs 26.1%/92; $p = 0.0354$) and high grade (14.9%/134 G1-2 vs 28.0%/75 G3; $p = 0.0767$, data not shown). In bladder cancer, the CK18 expression pattern varied between subgroups. Within 1,353 patients that were treated by cystectomy, CK18 expression was unrelated to pathological parameters and patient outcome, however.

Discussion

The standardized analysis of 14,579 cancers by IHC gives a comprehensive overview on CK18 staining in malignant tumors. The most valuable result of our study is a ranking order of CK18 positivity across a

broad range of tumor entities. The S-shaped curve of the CK18 expression frequencies found across 115 different tumor types reflects that frequent and intense CK18 immunostaining is commonly seen in cancers derived from CK18 positive normal cell types while most other tumors are often CK18 negative. 37 of 115 analyzed tumor entities (32.2%) showed CK18 positivity in >97% of cases. Sporadic negative cases in the range of ≤ 3% in these cancer types may well be caused by technical issues. Some unexpected negative staining always occurs in TMAs because not all tissues are properly fixed in all areas (48). Unequal immunostaining in tissues results in an immunostaining gradient across a tissue block and can result in false negative immunostaining, if TMA cores are taken from areas with poor reactivity (49).

The group of cancers with variable CK18 immunostaining results including significant fractions of patients with CK18 positive and CK18 negative cancers, is heterogeneous in nature. This category contains cancer types arising from CK18 positive precursor cells but showing CK18 downregulation in a fraction of cases, tumor types arising from CK18 negative precursor cells but undergoing CK18 upregulation in a fraction of cancers, neoplasia's derived from tissues with variable CK18 expression in benign precursors, and tumors with a mixed differentiation. The latter group contains tumors with a mixed glandular/squamous differentiation such as endometroid carcinomas of the uterus where adenomatous but not squamous epithelia stain positive as well as epithelial-mesenchymal tumors such as Malignant Muller Tumors, phyllodes tumor of the breast, teratoma of the testis or malignant mesothelioma. In these tumors, glandular epithelial but not mesenchymal tumor areas stain positive.

Cancers that markedly downregulate CK18 in a relevant fraction of cases include renal cell and breast cancers. True downregulation can easily be distinguished from artificial staining deficiency by presence of strongly staining normal cells in the same tissue spot. The analysis of larger cohorts of kidney and breast cancers for which clinical follow-up data were available identified significant associations of reduced CK18 immunostaining with unfavorable tumor phenotype and – in case of clear cell renal cell carcinoma – poor patient prognosis. These findings are consistent with earlier studies in breast cancer and may reflect a tendency towards a worse prognosis in cancer cells with an impaired cell differentiation (16, 50). That various cancers types that are by default poorly differentiated such as small cell carcinomas or anaplastic thyroid cancer showed lower CK18 positivity rates than their better differentiated counterparts is also consistent with the concept of a CK18 expression loss during tumor progression.

Squamous cell carcinomas are the best examples of epithelial tumors that are typically CK18 negative but can upregulate CK18. Even though CK18 immunostaining was not at all observed in any normal squamous epithelium samples from the lung, tonsil, skin, anal canal, oral cavity, or lip, a positive CK18 immunostaining was observed in 8 of 9 analyzed squamous cell carcinoma subtypes. That CK18 immunostaining was sometimes seen at high levels in these squamous cell carcinomas further demonstrates that these findings reflect true overexpression and not just a faint non-specific antibody binding. Our notion, that CK18 upregulation reflects aberrant differentiation or dedifferentiation in these cancers is supported by significant associations of elevated CK18 protein levels with high pT stage and

presence of nodal metastasis that could be identified in a combined analysis of our 230 squamous cell carcinomas with available clinico-pathological data. These findings fit with data from several earlier studies suggesting a link between CK18 positivity and unfavorable clinico-pathological features and outcome in squamous cell carcinomas of the lung, esophagus, oral cavity, and pharynx (18, 27, 40, 46, 47, 51, 52).

The role of CK18 is less clear in tumor entities derived from tissues with variable CK18 expression such as in liver and urinary bladder cancer. In our analysis of 1,353 urothelial carcinomas, 37% were completely negative and 20% of all cancers were considered strongly positive. That a marked difference in CK18 immunostaining was seen between pTa and pT2-4 urothelial carcinomas is consistent with the striking genomic differences between these tumor categories (summarized in (53)). The absence of a statistically significant impact of CK18 expression on clinico-pathological features and outcome of pT2-4 carcinomas treated by cystectomy argues against a functional role of CK18 for cancer progression. CK18 plays a role in various cellular processes, such as securing the structure of the cytoplasm and mitochondria that are not directly related to cancer aggressiveness (54). Considering the continuous increase of CK18 expression from basal and intermediate cells to the superficial and umbrella cells of the bladder epithelium, various levels of CK18 in cancer cells may also be related to the specific cell of origin.

Our data enable a comprehensive assessment of potential diagnostic applications of CK18 IHC. The close to 100% prevalence of CK18 expression in gastrointestinal cancers supports the concept of using CK18 measurement for metastasis detection (4, 18). The 100% positivity rate even in advanced prostate cancers suggests that cleaved CK18 measurement might be an alternative concept for disease monitoring in prostate cancer patients where PSA expression is markedly diminished. The most useful diagnostic application of CK18 IHC may be the distinction of seminomas from other germ cell tumors of the testis. Only 12 of 204 analyzed seminomas (6%) but all of 88 embryonal carcinomas and yolk sack tumors of the testis showed CK18 expression. This finding is in line with data from an RNA and protein expression study identifying CK18 as one of the strongest distinctors of seminomatous versus non-seminomatous testicular germ cell tumors (55). Pan-cytokeratin antibodies are often being used to exclude seminoma. Studies utilizing pan-cytokeratin staining in testicular cancer reported positive immunostaining in >20% (summarized in (56)) of seminomas, however. It appears possible that the limitation to an antibody targeting just one cytokeratin such as CK18 offers better specificity than seen for pan-cytokeratin antibodies.

Importantly, all prevalence's described in this study are specific to the reagents and the protocol used in our laboratory. It is almost certain, that the use of different antibodies, protocols and interpretation criteria have jointly caused highly diverse literature data on CK18 expression in cancer (summarized in Figure 5). It is well known, that different antibodies designed for the same target protein can vary to a large extent in their binding properties and that protocol modifications greatly impact the rate of immunostained cases (57).

Conclusions

Our data show that CK18 is consistently expressed in various epithelial cancers, especially adenocarcinomas. Both loss of CK18 expression in cancers derived from CK18 positive precursor cells and neo-expression in malignancies derived from CK18 positive precursors tend to be linked to unfavorable tumor phenotype and disease outcome. Distinction of seminomas from other germ cell tumors of the testis appears to be the strongest diagnostic application of CK18 IHC.

Abbreviations

Akt1 Serine/Threonine-protein kinase

CK18 Cytokeratin 18

ER Estrogen Receptor

ERK extracellular signal-regulated kinase

IHC Immunohistochemistry

ISUP International Society of Urological Pathology

MAPK mitogen-activated protein kinase

PR Progesterone Receptor

TMA Tissue microarray

UICC International Union Against Cancer

Declarations

Ethical approval

The usage of archived diagnostic left-over tissues for manufacturing of tissue microarrays, their analysis for research purposes and patient data analysis has been approved by local laws (HmbKHG, §12,1) and by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

Consent for publication

Not applicable

Availability of supporting data

All data generated or analyzed during this study are included in this published article.

Competing interests

The Institute of Pathology of the UKE receives royalties on the sale of CK18 clone MSVA-118 from MS Validated Antibodies GmbH (owned by a family member of GS).

Funding

No funding

Authors' contribution

Anne Menz, Ronald Simon, Martina Kluth, Doris Höflmayer, Katharina Möller, Sören Weidemann, Till Clauditz, Claudia Hube-Magg, Andrea Hinsch contributed to conception, design, data collection, data analysis and manuscript writing.

Anne Menz; Timo Weitbrecht, Franziska Büscheck, Andreas Luebke, Christoph Fraune, Christian Bernreuther: immunohistochemistry analysis

Rainer Krech, Till Krech, Andreas Marx: conception and design, collection of samples

Patrick Lebock, David Dum, Stefan Steurer, Ria Uhlig, Sarah Minner, Eike Burandt: data collection and data analysis

Anne Menz, Guido Sauter, Waldemar Wilczak, Ronald Simon: study supervision

Acknowledgments

We are grateful to Melanie Witt, Inge Brandt, Maren Eisenberg, Christina Möller-Koop, and Sünje Seekamp for excellent technical assistance.

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

Figures

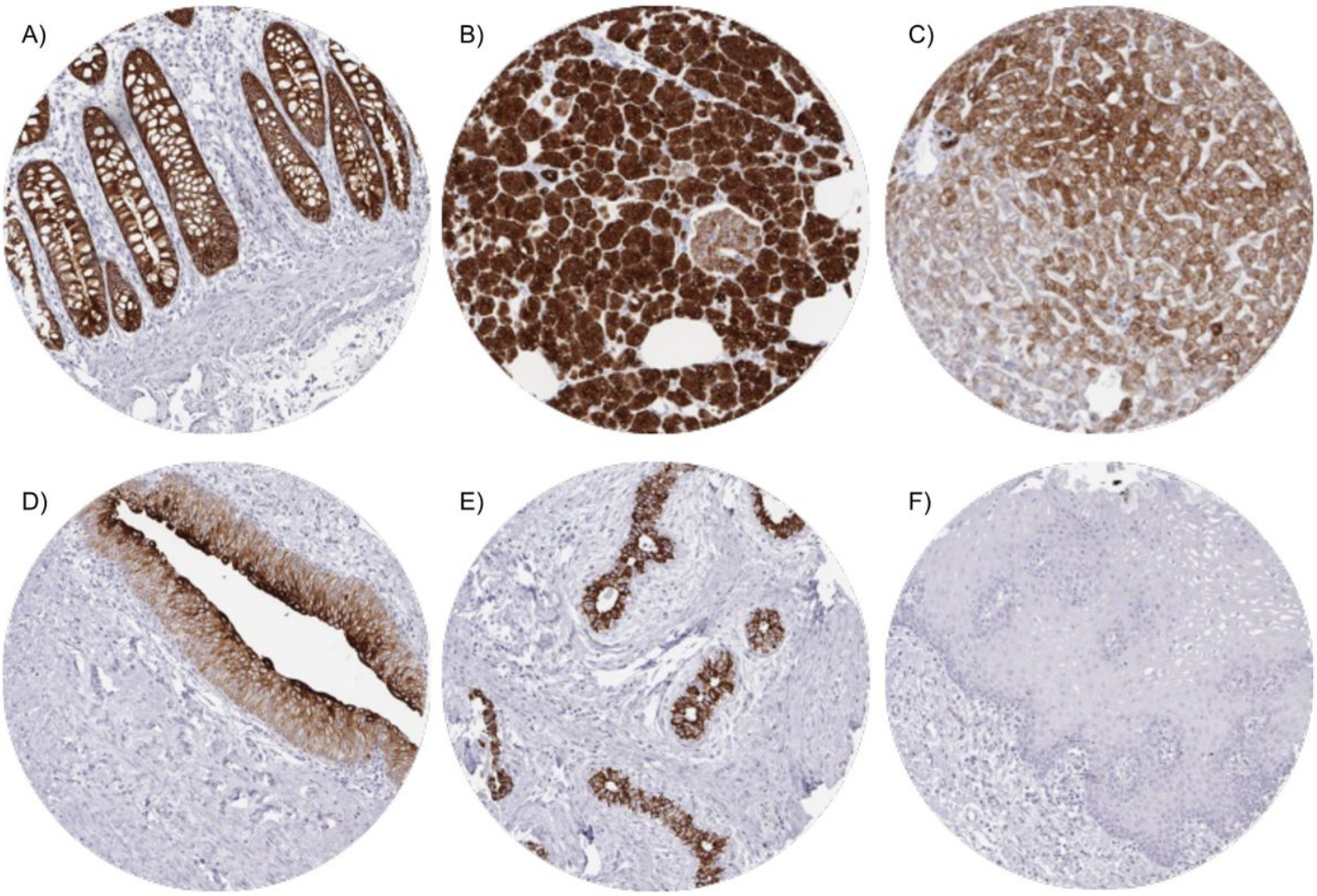

Figure 1

Cytokeratin 18 (CK18) expression in normal tissues. The images show strong CK18 staining in epithelial cells from rectum (A) and pancreas (B), a zonal staining variability in the liver (C), strongly positive umbrella cells and a gradually decreasing staining intensity from superficial to basal urothelial cells in the bladder (D), strong positivity in acinus cells but absent staining in basal cells of the breast epithelium (E) and a complete lack of staining in squamous epithelium of the oral mucosa (F).

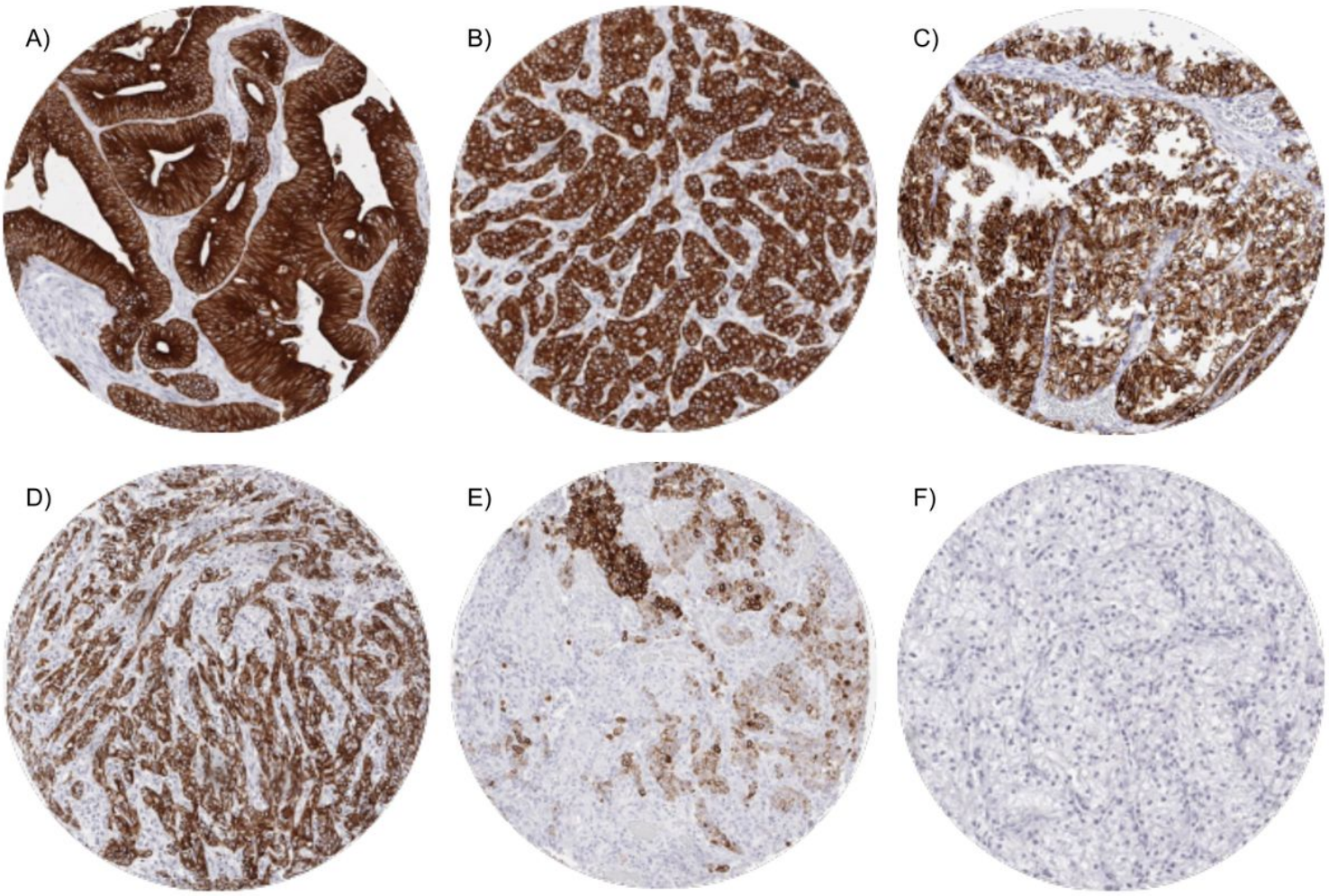

Figure 2

Cytokeratin 18 (CK18) expression in tumors. The images show diffuse strong CK18 staining in a colorectal carcinoma (A), a breast cancer NST (B), a clear cell carcinoma of the kidney (C), and a squamous cell carcinoma of the cervix uteri (D). CK18 immunostaining is focal in a squamous cell carcinoma of the larynx (E) and absent in another renal clear cell carcinoma (F)

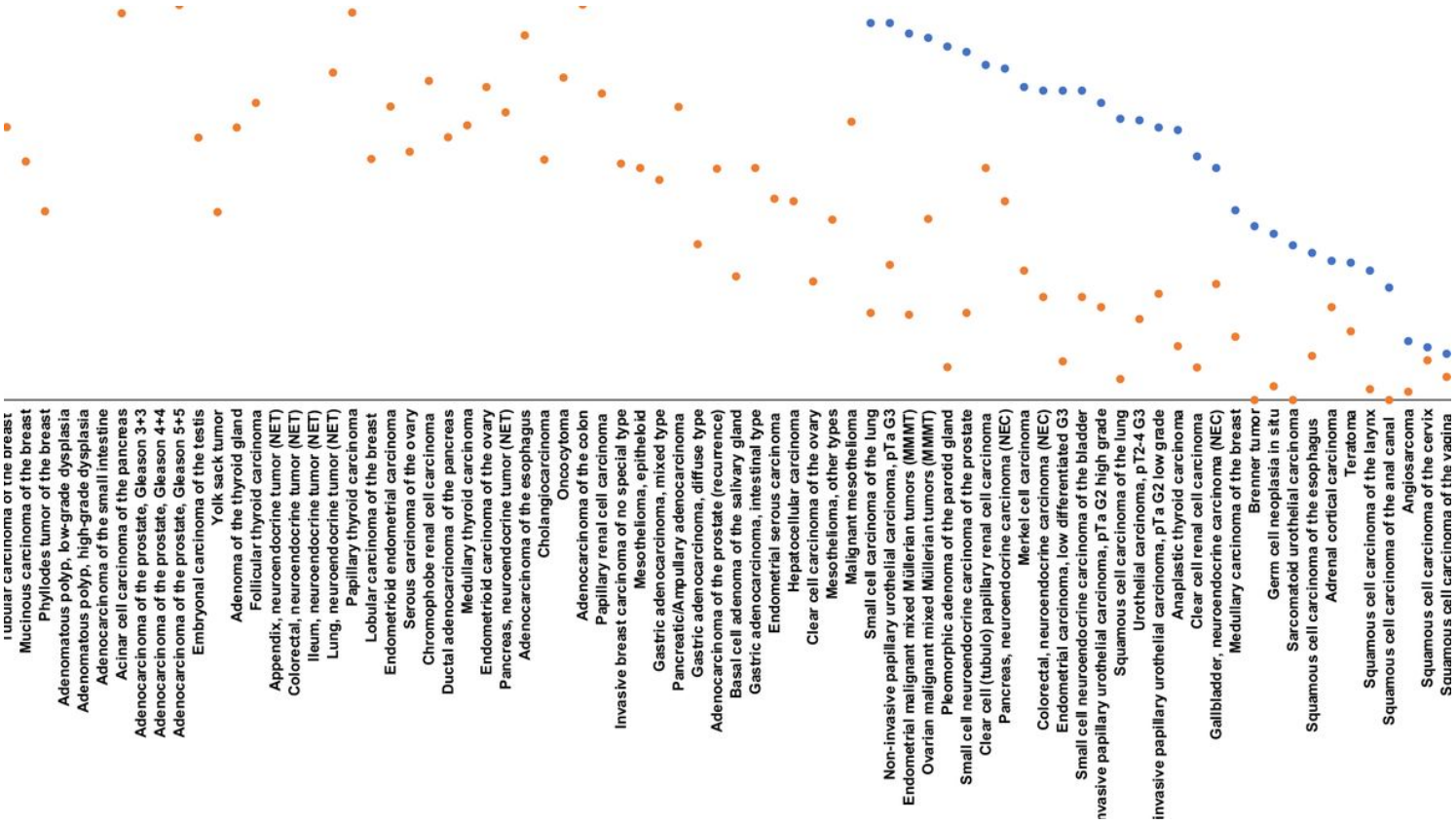

Figure 3

Ranking order of Cytokeratin 18 (CK18) immunostaining in cancers. Both the frequency of positive cases (blue dots) and the frequency of strongly positive cases (orange dots) are shown. The conspicuously low rate of strongly positive Whartin tumors is due to the fact, that only basal cells react with CK18 resulting in a low overall percentage of positive cells. 25 additional tumor entities without any CK18 positive cases are not shown due to space restrictions.

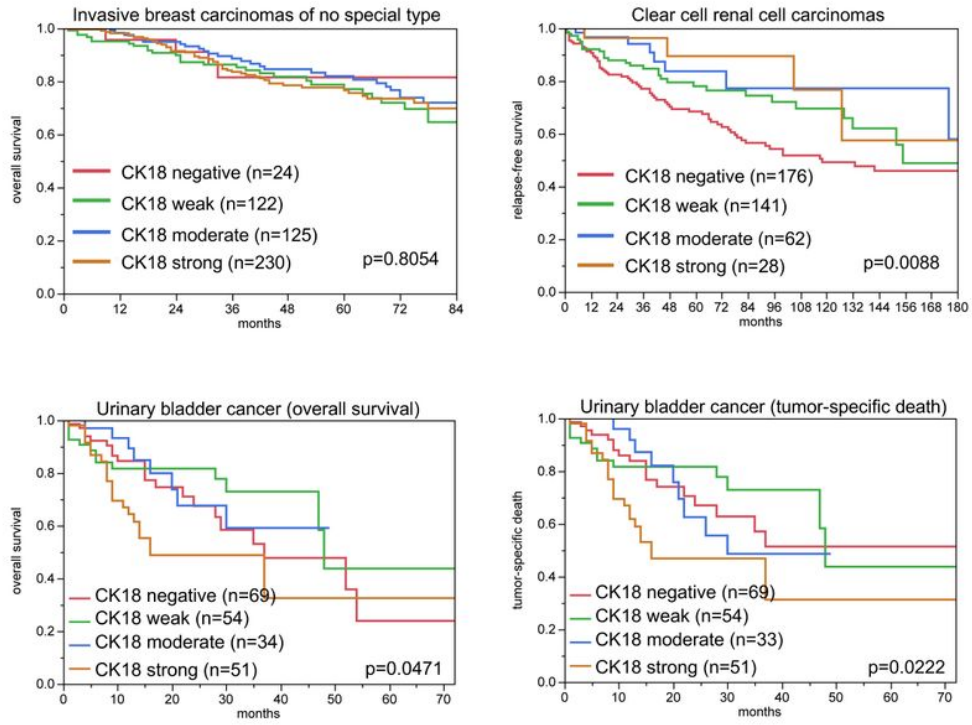

Figure 4

Cytokeratin 18 (CK18) immunostaining and patient prognosis. All bladder cancer patients had at least pT2 cancers and were treated by cystectomy.

Figure 5

Graphical comparison of Cytokeratin 18 data from this study (x) in comparison with the previous literature. Orange dots are used for studies involving ≤20 cases, green dots are used for studies >20 cases, blue dots are from Chu and Weiss 2002 (Review)(58). All studies are quoted in the list of references.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CK18MTATable1.xlsx
- CK18MTATable2.xlsx