High prevalence of p16 staining in malignant tumors

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Abstract

p16 (CDKN2A) is a member of the INK4 class of cell cycle inhibitors, which is often dysregulated in cancer. However, the prevalence of p16 expression in different cancer types is controversial. 15,783 samples from 124 different tumor types and 76 different normal tissue types were analyzed by immunohistochemistry in a tissue microarray format. p16 was detectable in 5,292 (45.0%) of 11,759 interpretable tumors. Except from adenohypophysial islets of Langerhans, p16 staining was largely absent in normal tissues. In cancer, highest positivity rates were observed in uterine cervix squamous cell carcinomas (94.4%), non-invasive papillary urothelial carcinoma, pTaG2 (100%), Merkel cell carcinoma (97.7%), and small cell carcinomas of various sites of origin (54.5%-100%). All 124 tumor categories showed at least occasional p16 immunostaining. Comparison with clinico-pathological data in 128 vulvar, 149 endometrial, 295 serous ovarian, 396 pancreatic, 1365 colorectal, 284 gastric, and 1245 urinary bladder cancers, 910 breast carcinomas, 620 clear cell renal cell carcinomas, and 414 testicular germ cell tumors revealed only few statistically significant associations. Comparison of human papilloma virus (HPV) status and p16 in 497 squamous cell carcinomas of different organs revealed HPV in 80.4% of p16 positive and in 20.6% of p16 negative cancers (p<0.0001). It is concluded, that a positive and especially strong p16 immunostaining is a feature for malignancy which may be diagnostically useful in lipomatous, urothelial and possibly other tumors. The imperfect association between p16 immunostaining and HPV infection with high variability between different sites of origin challenges the use of p16 immunohistochemistry as a surrogate for HPV positivity, except in tumors of cervix uteri and the penis.
**Introduction**

The p16 protein is encoded by the *cyclin dependent kinase inhibitor 2A* gene (*CDKN2A*, syn. *MTS-1, INK4a* or *p16INK4a*) located at chromosome 9p21 [1]. p16 inhibits cell cycle progression from G1 to S phase [2] through binding and inactivating cyclin dependent kinases CDK4 and CDK6 [3]. In its cell cycle inhibiting function, p16 interplays with the retinoblastoma (RB1) and the p53 tumor suppressor genes. In case of an inactivation of p53 or RB1 and especially in case of inactivation of both proteins, p16 can be markedly upregulated. Accordingly, a particularly strong up-regulation is seen in human papilloma virus (HPV) infected cells, where both p53 and RB1 are inactivated by the HPV proteins E6 and E7 [4,5].

More than 3,000 studies have employed immunohistochemistry to study the role of p16 expression in normal and neoplastic tissues. Due to its strong overexpression in HPV infected cells, p16 immunohistochemistry is routinely used in diagnostic pathology as a surrogate parameter for HPV infection and a marker for HPV related anogenital and oropharyngeal neoplasia. The role of p16 expression in other cancer types is less clear. The rate of reported p16 positivity is highly variable for many tumors. For example, the fraction of p16 positive cases ranged from 43% to 100% in squamous cell carcinoma of the cervix [6,7], 9% to 98% in colorectal adenocarcinoma [8–10], 12% to 64% in hepatocellular carcinoma [11,12], 4% to 96% in malignant melanoma [13–15], 20% to 62% in mesothelioma [16–18], and 0% to 100% in liposarcomas [19–21]. Although many studies have described a prognostic role of reduced or increased p16 expression, these results have often not been confirmed by others. In several cancer types including breast, prostate, ovarian, and colorectal cancer, both reduced expression [22–25] and overexpression [26–29] have been reported to be linked to poor prognosis. Altogether, these conflicting data are likely to be caused by the use of different antibodies, immunostaining protocols, and criteria to determine p16 positivity in these studies.

To better understand the role of p16 immunohistochemistry in different tumor types, a comprehensive study analyzing a large number of neoplastic and non-neoplastic tissues under highly standardized conditions is needed. We thus analyzed p16 expression in more than 15,000 tumor tissue samples from 124 different tumor types and subtypes as well as 76 non-neoplastic tissue types by immunohistochemistry in a tissue microarray (TMA) format.

**Materials and methods**

**Tissue Microarrays (TMAs)**

To study p16 expression in normal and neoplastic human tissues, preexisting TMAs containing 15,783 primary tumors from 124 tumor types and subtypes as well as 608 samples of 76 different normal tissues were used. Detailed histopathological data on grade, pT and pN status were available for 7,598 cancers (invasive breast carcinoma of no special type, colorectal carcinoma, endometroid endometrial carcinoma, clear cell renal cell carcinoma, serous high grade ovarian carcinoma, adenocarcinoma of the pancreas, adenocarcinoma of the stomach, germ cell tumors, carcinoma of the vulva and urinary bladder carcinoma). Clinical follow up data were available for 254 patients who had undergone cystectomy for muscle invasive (pT≥2) urinary bladder cancer (median follow-up time = 14 (range 1–77) months) and 978 patients with invasive breast carcinoma of no special type (median follow-up time = 50 (range 1–88) months). The composition of both normal and cancer TMAs is described in detail in the results section. All samples were derived from the archives of the Institute of Pathology, University Hospital of Hamburg, Germany, the Institute of Pathology, Clinical Center Osnabrueck, Germany, and the Department of Pathology, Academic Hospital Fuert, Germany. Tissues were fixed in 4% buffered formalin and then embedded in paraffin. TMA tissue spot
diameter was 0.6 mm. Informed patient consent was not required for this retrospective study. The use of anonymized 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 with xylol, rehydrated through a graded alcohol series and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at 121°C in pH 9 DakoTarget Retrieval Solution™ (Agilent, CA, USA; #S2367). Endogenous peroxidase activity was blocked with Dako Peroxidase Blocking Solution™ (Agilent, CA, USA; #52023) for 10 minutes. Primary antibody specific against p16 protein (rabbit recombinant clone MSVA-016R; MS Validated Antibodies GmbH, Hamburg, Germany) was applied at 37°C for 60 minutes at a dilution of 1:150. Bound antibody was then visualized using the EnVision Kit™ (Agilent, CA, USA; #K5007) according to the manufacturer’s directions. The sections were counterstained with haemalaun. 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 as negative. Tumors with 1+ staining intensity in ≤70% of cells or 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 were considered strongly positive.

**HPV polymerase chain reaction (PCR) and sequencing**

HPV status was analyzed in a subset of 551 squamous cell carcinomas including 80 oral, 60 pharyngeal, 60 laryngeal, 80 cervical, 30 vaginal, 80 vulvar, 80 penile, 40 skin and 41 anal canal tumors. Detection of HPV-DNA was performed on formalin-fixed, paraffin-embedded tumor specimens. One 4μm microtome section was taken from each sample for DNA extraction using the Maxwell® RSC DNA FFPE Kit (Promega, Fitchburg, WI, USA) according to the manufacturer’s protocol. Suitability of the isolated DNA for PCR analysis was verified by amplification of a β-globin sequence with primers generating an amplicon of 217 bp (forward 5'–GCCATCCTAAAGGCACCCGAC-3' and reverse 5'–TGGGCTAGTGGACGAGAGAAGA-3'). Detection of HPV was performed using primers HPV-GP 6+ (5'–GAAAAAATAACTGTAAATC ATATTC-3`) and HPV-GP 5+ (5'–TTTGTACTGTGAGAGATAC-3`) which generate amplicons ranging between 139–145 bp. The thermocycler protocol included initial denaturation at 95°C for 10 min, followed by 34 cycles of 95°C for 90 sec, 55°C for 90 sec and 72°C for 120 sec, and a final extension step at 72°C for 7 min. PCR products were visualized by standard agarose gel electrophoresis. Samples with negative β-globin PCR were excluded from further analysis. Samples with positive β-globin PCR but negative HPV PCR were reported as HPV-negative. Samples with positive β-globin PCR and positive HPV PCR were reported as HPV-positive and subjected to bidirectional Sanger sequencing employing the Genetic Analyzer 3130 xl device (Applied Biosystems, Foster City, CA, USA) using primers GP 5+ and GP 6+. Sequences were analyzed with NCBI’s Basic Local Alignment Search Tool (BLAST) [30] to determine the HPV type.
Statistics
Statistical calculations were performed with JMP14 software (SAS Institute Inc., NC, USA). Contingency tables and the chi²-test were performed to search for associations between p16 and tumor phenotype. Survival curves were calculated according to Kaplan-Meier. The Log-Rank test was applied to detect significant differences between groups. A p-value of $\leq 0.05$ was considered as statistically significant.

Results

Technical issues
A total of 11,759 (74.5%) of 15,783 tumor samples and 405 (66.6%) of 608 normal samples were interpretable for p16 immunostaining in our TMA analysis. Non-interpretable samples (4,024; 25.5%) either lacked unequivocal tumor cells or were absent on the TMA.

p16 in normal tissue
p16 staining was strongest in islets of Langerhans of the pancreas (Fig 1A) and in a large fraction of cells in the adenohypophysis (Fig 1B). Positive staining was also found in a fraction of cells of corpuscles of Hassall's of thymus (Fig 1C), scattered adrenocortical cells (Fig 1D), and endothelial cells of blood vessels in a normal placenta (Fig 1E) and in an otherwise p16 negative clear cell carcinoma of the kidney (Fig 1F).

p16 immunostaining was absent in endothelium and media of the aorta, the heart, striated muscle, tongue muscle, myometrium of the uterus, muscular wall of the appendix, esophagus, stomach, ileum, colon descendens, kidney pelvis, and urinary bladder, corpus spongiosum of the penis, ovarian stroma, fat, skin, hair follicle and sebaceous glands of the skin, oral mucosa of the lip, oral cavity, surface epithelium of the tonsil, transitional mucosa and skin of the anal canal, ectocervix, squamous epithelium of the esophagus, urothelium of the kidney pelvis and urinary bladder, amnion and chorion of the mature placenta, spleen, antrum and corpus of the stomach, epithelium of the gallbladder, liver, Brunner gland of the duodenum, cortex and medulla of the kidney, seminal vesicle, epididymis, testis, lung, endocervix, mucosa of the fallopian tube, decidua of the early placenta, in the cerebellum, and white and grey matter of the cerebrum.

p16 immunostaining in tumor cells
Positive p16 immunostaining was detectable in 5,292 (45.0%) of the 11,759 analyzable tumors, including 3,152 (26.8%) with weak, 683 (5.8%) with moderate, and 1,457 (12.4%) with strong immunostaining. The staining pattern was heterogenous and included cases with variable percentage of positive muscle, myometrium of the uterus, muscular wall of the appendix, esophagus, stomach, ileum, colon descendens, kidney pelvis, and urinary bladder, corpus spongiosum of the penis, ovarian stroma, fat, skin, hair follicle and sebaceous glands of the skin, oral mucosa of the lip, oral cavity, surface epithelium of the tonsil, transitional mucosa and skin of the anal canal, ectocervix, squamous epithelium of the esophagus, urothelium of the kidney pelvis and urinary bladder, amnion and chorion of the mature placenta, spleen, antrum and corpus of the stomach, epithelium of the gallbladder, liver, Brunner gland of the duodenum, cortex and medulla of the kidney, seminal vesicle, epididymis, testis, lung, endocervix, mucosa of the fallopian tube, decidua of the early placenta, in the cerebellum, and white and grey matter of the cerebrum.

p16 immunostaining, tumor phenotype and prognosis
A comparison of p16 expression with pT, pN, histologic grade, and patient prognosis in 128 analyzable vulvar carcinomas, 149 endometrioid endometrial carcinomas, 295 serous high grade ovarian carcinomas, 910 invasive breast carcinomas of no special type, 1245 urinary
Fig 1. p16 immunostaining in non-neoplastic tissue. The panels show a nuclear and cytoplasmic p16 staining of a fraction of cells of pancreatic islets of Langerhans (A), a large fraction of epithelial cells in the adenohypophysis (B), a fraction of cells of corpuscles of Hassall’s of thymus (C), and of scattered adrenocortical cells (D). A p16 positivity is also seen in endothelial cells of blood vessels in a normal placenta (E) and in an otherwise p16 negative clear cell carcinoma of the kidney (F).

https://doi.org/10.1371/journal.pone.0262877.g001
**Fig 2. p16 immunostaining of tumors and related normal tissues.** In the pancreas, a moderate p16 immunostaining is regularly seen in a subset of islet cells and only occasionally occurs in few scattered cells of excretory ducts (A), but p16 expression can be strong in cases of ductal adenocarcinoma (B) and of neuroendocrine carcinoma (C). In normal lymphatic tissues, a weak p16 staining occurs in germinal centre macrophages and in some scattered lymphocytes (D) but a strong staining is seen in neoplastic cells of some Hodgkin’s (E) and diffuse large B-cell lymphomas (F). In the stomach, few normal epithelial cells...
may show p16 staining (G) while p16 staining can be strong in gastric adenocarcinoma (H). In the esophagus, few cells with weak to moderate p16 staining can be found in some samples of normal squamous epithelium (I) but p16 staining can be intense in squamous cell carcinoma (J). p16 immunostaining is usually absent in normal myometrium (K), fat (L), urothelium (M), and cervical squamous epithelium (N) while staining can be intense in tumors derived from these tissues such as leiomyosarcoma of the uterus (O), liposarcoma (P), urethelial carcinoma (Q) as well as adenocarcinoma (R) and squamous cell carcinoma (S) of the uterine cervix. A similarly strong p16 staining can also be seen in other squamous cell carcinomas such as of the skin (T).

https://doi.org/10.1371/journal.pone.0262877.g002

bladder carcinomas, 620 clear cell renal cell carcinomas, 414 germ cell tumors of the testis, 284 gastric adenocarcinomas, 396 pancreatic adenocarcinomas and 1365 colorectal adenocarcinomas revealed only few statistically significant associations (Table 2). Positive p16 immunostaining was associated with high pT category in urinary bladder carcinoma (p < 0.0001) and gastric adenocarcinoma (p = 0.0212), and with high grade in invasive breast carcinoma of no special type (p < 0.001). In colorectal adenocarcinoma, a significant association was found between p16 positivity (at least weak immunostaining) and MMR (mismatch repair) status, with a higher percentage of tumors showing positive p16 staining in the MMR-proficient group (56%) than in the MMR-deficient group (27%, p < 0.0001). In cohorts of 502 invasive breast cancers and 151 urothelial carcinomas with clinical follow-up data, p16 immunostaining was unrelated to overall survival (Fig 4).

p16 immunostaining and HPV-status

HPV analysis of 535 squamous cell carcinomas of different sites of origin revealed 244 HPV positive cases (45.6%). Among these, 98.0% were high risk type (HPV type 16, 18, 33, 35, 45, 58), 1.6% intermediate risk type (HPV type 56, 67, 73) and 0.4% low risk type (HPV type 6). The comparison of HPV status and p16 staining revealed a strong but not perfect association between these parameters (Table 3). HPV was detected in 80.4% of 163 tumors with strong, 62.3% of 42 tumors with moderate, 25.9% of 42 tumors with weak p16 positivity, but also in 20.6% of 250 p16 negative cancers (p < 0.0001). The association between p16 expression and HPV status varied between the organs of tumor origin and was particularly strong in squamous cell carcinomas of the cervix, squamous cell carcinoma of the penis and squamous cell carcinoma of the pharynx (p < 0.0001 each). The statistical association between p16 expression and HPV status was particularly weak in squamous cell carcinomas of the larynx and of the vulva. It is of note, however, that both HPV negative cases with strong p16 positivity, and HPV positive cases with negative p16 staining were found in almost all tumor entities. Only cervical and skin cancer lacked HPV positive but p16 negative cases.

Discussion

The successful analysis of 11,759 cancers and 76 normal tissue types revealed that—as compared to normal tissues—p16 is often upregulated in cancers. While the normal tissue analysis demonstrated a moderate to strong p16 immunostaining in only few tissues, a strong p16 positivity was found in many tumors. Although p16 is a known tumor suppressor gene, upregulation can occur directly as a consequence of an altered state of the interaction partner pRb [31] or indirectly through pathway crosstalk with p53 (reviewed in [32]). Considering that p53 is the most frequently mutated tumor suppressor gene in cancer [33], that p53 inactivation can also occur in the absence of p53 gene mutations (reviewed in [34]), and that alterations of Rb (reviewed in [35]) and other p16 interaction partners such as CDK4 (cyclin dependent kinase 4) [36] are also common in cancer, the high rate of p16 upregulation is not a surprise. Our data also revealed a correlation between p16 staining and microsatellite instability in colorectal adenocarcinoma. In the microsatellite-stable group a higher percentage of tumors showed positive p16 staining than in the microsatellite-_instable group. This may be explained by the
| Tumor entity | on TMA (n) | analyzable (n) | negative (%) | weak (%) | moderate (%) | strong (%) | positive (%) |
|--------------|------------|----------------|---------------|----------|--------------|------------|--------------|
| **Tumors of the skin** |            |                |               |          |              |            |              |
| Pilomatrixoma | 35         | 28             | 32.1          | 64.3     | 3.6          | 0.0        | 67.9         |
| Basal cell carcinoma | 88         | 67             | 9.0           | 68.7     | 20.9         | 1.5        | 91.0         |
| Benign nevus | 29         | 25             | 4.0           | 60.0     | 20.0         | 16.0       | 96.0         |
| Squamous cell carcinoma of the skin | 90         | 85             | 64.7          | 20.0     | 5.9          | 9.4        | 35.3         |
| Malignant melanoma | 48         | 41             | 53.7          | 19.5     | 19.5         | 7.3        | 46.3         |
| Merkel cell carcinoma | 46         | 44             | 2.3           | 2.3      | 9.1          | 86.4       | 97.7         |
| **Tumors of the head and neck** |            |                |               |          |              |            |              |
| Squamous cell carcinoma of the larynx | 110        | 90             | 76.7          | 13.3     | 4.4          | 5.6        | 23.3         |
| Squamous cell carcinoma of the pharynx | 60         | 52             | 51.9          | 7.7      | 7.7          | 32.7       | 48.1         |
| Oral squamous cell carcinoma (floor of the mouth) | 130        | 114            | 76.3          | 7.9      | 3.5          | 12.3       | 23.7         |
| Pleomorphic adenoma of the parotid gland | 50         | 33             | 6.1           | 81.8     | 12.1         | 0.0        | 93.9         |
| Warthin tumor of the parotid gland | 49         | 41             | 2.4           | 87.8     | 9.8          | 0.0        | 97.6         |
| Basal cell adenoma of the salivary gland | 15         | 13             | 0.0           | 100.0    | 0.0          | 0.0        | 100.0        |
| **Tumors of the lung, pleura and thymus** |            |                |               |          |              |            |              |
| Squamous cell carcinoma of the lung | 77         | 40             | 82.5          | 5.0      | 10.0         | 2.5        | 17.5         |
| Adenocarcinoma of the lung | 200        | 107            | 58.9          | 27.1     | 10.3         | 3.7        | 41.1         |
| Small cell carcinoma of the lung | 20         | 16             | 18.8          | 12.5     | 63.0         | 0.0        | 81.3         |
| Mesothelioma, epitheloid | 39         | 30             | 76.7          | 23.3     | 0.0          | 0.0        | 23.3         |
| Mesothelioma, other types | 76         | 63             | 63.5          | 12.7     | 0.0          | 42.9       | 55.6         |
| Thymoma | 29         | 25             | 36.0          | 60.0     | 4.0          | 0.0        | 64.0         |
| **Tumors of the female genital tract** |            |                |               |          |              |            |              |
| Squamous cell carcinoma of the vagina | 78         | 73             | 37.0          | 11.0     | 9.6          | 42.5       | 63.0         |
| Squamous cell carcinoma of the vulva | 130        | 116            | 60.3          | 12.1     | 8.6          | 19.0       | 39.7         |
| Squamous cell carcinoma of the cervix | 130        | 125            | 5.6           | 4.0      | 11.2         | 79.2       | 94.4         |
| Adenocarcinoma of the cervix uteri | 50         | 48             | 10.4          | 52.1     | 22.9         | 14.6       | 89.6         |
| Endometrioid endometrial carcinoma | 236        | 195            | 19.0          | 63.6     | 12.3         | 5.1        | 81.0         |
| Endometrial serous carcinoma | 82         | 69             | 13.0          | 26.1     | 20.3         | 40.6       | 87.0         |
| Carcinosarcoma of the uterus | 48         | 39             | 10.3          | 20.5     | 38.5         | 30.8       | 89.7         |
| Endometrial carcinoma, high grade, G3 | 13         | 8              | 12.5          | 62.5     | 12.5         | 12.5       | 87.5         |
| Endometrial clear cell carcinoma | 8          | 5              | 0.0           | 80.0     | 20.0         | 0.0        | 100.0        |
| Endometrial stromal sarcoma | 12         | 12             | 75.0          | 16.7     | 0.0          | 8.3        | 25.0         |
| Endometrioid carcinoma of the ovary | 115        | 89             | 18.0          | 47.2     | 19.1         | 15.7       | 82.0         |
| Serous carcinoma of the ovary | 567        | 446            | 11.7          | 24.0     | 16.4         | 48.0       | 88.3         |
| Mucinous carcinoma of the ovary | 97         | 66             | 78.8          | 18.2     | 3.0          | 0.0        | 21.2         |
| Clear cell carcinoma of the ovary | 54         | 38             | 36.8          | 47.4     | 15.8         | 0.0        | 63.2         |
| Carcinosarcoma of the ovary | 47         | 36             | 22.2          | 30.6     | 19.4         | 27.8       | 77.8         |
| Brenner tumor | 9          | 9              | 22.2          | 66.7     | 11.1         | 0.0        | 77.8         |
| **Tumors of the breast** |            |                |               |          |              |            |              |
| Invasive breast carcinoma of no special type | 1387       | 960            | 61.1          | 29.5     | 3.6          | 5.7        | 38.9         |
| Lobular carcinoma of the breast | 294        | 168            | 67.3          | 30.4     | 1.2          | 1.2        | 32.7         |
| Medullary carcinoma of the breast | 26         | 22             | 22.7          | 13.6     | 13.6         | 50.0       | 77.3         |
| Tubular carcinoma of the breast | 27         | 14             | 28.6          | 71.4     | 0.0          | 0.0        | 71.4         |
| Mucinous carcinoma of the breast | 58         | 30             | 53.3          | 40.0     | 6.7          | 0.0        | 46.7         |
| Phylloides tumor of the breast | 50         | 42             | 7.1           | 71.4     | 14.3         | 7.1        | 92.9         |
| **Tumors of the digestive system** |            |                |               |          |              |            |              |
| Adenomatous polyp, low-grade dysplasia | 50         | 49             | 53.1          | 44.9     | 2.0          | 0.0        | 46.9         |
| Adenomatous polyp, high-grade dysplasia | 50         | 49             | 26.5          | 63.3     | 10.2         | 0.0        | 73.5         |

(Continued)
| Tumor Entity                                      | Analyzable (n) | Negative (%) | Weak (%) | Moderate (%) | Strong (%) | Positive (%) |
|--------------------------------------------------|----------------|--------------|----------|--------------|------------|--------------|
| Adenocarcinoma of the colon                      | 1882           | 48.2         | 48.6     | 2.6          | 0.6        | 51.8         |
| Adenocarcinoma of the small intestine             | 10             | 80.0         | 0.0      | 10.0         | 10.0       | 20.0         |
| Gastric adenocarcinoma, diffuse type              | 176            | 54.5         | 28.7     | 10.9         | 5.9        | 45.5         |
| Gastric adenocarcinoma, intestinal type           | 174            | 59.1         | 23.6     | 7.1          | 10.2       | 40.9         |
| Gastric adenocarcinoma, mixed type                | 62             | 62.2         | 28.9     | 0.0          | 8.9        | 37.8         |
| Adenocarcinoma of the esophagus                   | 133            | 87.7         | 1.8      | 1.8          | 8.8        | 12.3         |
| Squamous cell carcinoma of the esophagus          | 124            | 81.8         | 0.0      | 4.5          | 13.6       | 18.2         |
| Squamous cell carcinoma of the anal canal         | 91             | 24.1         | 4.6      | 10.3         | 60.9       | 75.9         |
| Cholangiocarcinoma                                | 130            | 73.5         | 20.6     | 3.9          | 2.0        | 26.5         |
| Hepatocellular carcinoma                         | 50             | 94.0         | 6.0      | 0.0          | 0.0        | 6.0          |
| Ductal adenocarcinoma of the pancreas             | 612            | 81.2         | 13.2     | 3.2          | 2.4        | 18.8         |
| Pancreatic/Ampullary adenocarcinoma               | 89             | 69.2         | 21.2     | 5.8          | 3.8        | 30.8         |
| Acinar cell carcinoma of the pancreas             | 13             | 75.0         | 8.3      | 8.3          | 8.3        | 25.0         |
| Gastrointestinal stromal tumor (GIST)             | 50             | 35.6         | 40.0     | 8.9          | 15.6       | 64.4         |
| Tumors of the urinary system                      |                |              |          |              |            |              |
| Non-invasive papillary urothelial carcinoma, pTa G2 low grade | 177            | 0.0          | 0.0      | 2.6          | 97.4       | 100.0        |
| Non-invasive papillary urothelial carcinoma, pTa G2 high grade | 141            | 0.0          | 0.9      | 8.5          | 90.6       | 100.0        |
| Non-invasive papillary urothelial carcinoma, pTa G3 | 187            | 9.8          | 6.1      | 15.2         | 68.9       | 90.2         |
| Urothelial carcinoma, pT2-4 G3                     | 1214           | 50.7         | 20.4     | 10.1         | 18.9       | 49.3         |
| Small cell neuroendocrine carcinoma of the bladder | 18             | 0.0          | 0.0      | 0.0          | 100.0      | 100.0        |
| Sarcomatoid urothelial carcinoma                  | 25             | 54.2         | 12.5     | 0.0          | 33.3       | 45.8         |
| Clear cell renal cell carcinoma                   | 858            | 98.2         | 1.7      | 0.2          | 0.0        | 1.8          |
| Papillary renal cell carcinoma                    | 255            | 67.2         | 31.3     | 1.6          | 0.0        | 32.8         |
| Clear cell (tubulo) papillary renal cell carcinoma | 21             | 92.9         | 7.1      | 0.0          | 0.0        | 7.1          |
| Chromophobe renal cell carcinoma                  | 131            | 77.7         | 22.3     | 0.0          | 0.0        | 22.3         |
| Oncocytoma                                        | 177            | 95.5         | 4.5      | 0.0          | 0.0        | 4.5          |
| Tumors of the male genital organs                 |                |              |          |              |            |              |
| Adenocarcinoma of the prostate, Gleason 3+3       | 83             | 92.1         | 6.3      | 1.6          | 0.0        | 7.9          |
| Adenocarcinoma of the prostate, Gleason 4+4       | 80             | 67.2         | 31.3     | 1.6          | 0.0        | 32.8         |
| Adenocarcinoma of the prostate, Gleason 5+5       | 85             | 63.9         | 36.1     | 0.0          | 0.0        | 36.1         |
| Adenocarcinoma of the prostate (recurrence)       | 330            | 35.6         | 57.0     | 2.5          | 4.9        | 64.4         |
| Small cell neuroendocrine carcinoma of the prostate | 17             | 63           | 18.8     | 12.5         | 62.5       | 93.8         |
| Seminoma                                          | 620            | 90.3         | 9.3      | 0.2          | 0.2        | 9.7          |
| Embryonal carcinoma of the testis                 | 50             | 82.9         | 14.6     | 2.4          | 0.0        | 17.1         |
| Yolk sack tumor                                   | 50             | 80.6         | 19.4     | 0.0          | 0.0        | 19.4         |
| Teratoma                                          | 50             | 88.9         | 5.6      | 2.8          | 2.8        | 11.1         |
| Squamous cell carcinoma of the penis              | 80             | 49.3         | 8.0      | 4.0          | 38.7       | 50.7         |
| Tumors of endocrine organs                        |                |              |          |              |            |              |
| Adenoma of the thyroid gland                      | 50             | 83.0         | 17.0     | 0.0          | 0.0        | 17.0         |
| Papillary thyroid carcinoma                       | 114            | 90.6         | 9.4      | 0.0          | 0.0        | 9.4          |
| Follicular thyroid carcinoma                      | 392            | 70.9         | 26.4     | 2.4          | 0.3        | 29.1         |
| Medullary thyroid carcinoma                       | 158            | 87.7         | 11.5     | 0.8          | 0.0        | 12.3         |
| Anaplastic thyroid carcinoma                      | 107            | 76.3         | 22.5     | 1.3          | 0.0        | 23.8         |

(Continued)
| Tumor Entity | Analyzable (n) | Negative (%) | Weak (%) | Moderate (%) | Strong (%) | Positive (%) |
|--------------|----------------|--------------|----------|--------------|------------|--------------|
| Adrenal cortical adenoma | 45 | 66.7 | 2.4 | 7.1 | 23.8 | 33.3 |
| Adrenal cortical carcinoma | 26 | 26.9 | 23.1 | 19.2 | 30.8 | 73.1 |
| Phaeochromocytoma | 50 | 58.0 | 38.0 | 4.0 | 0.0 | 42.0 |
| Appendix, neuroendocrine tumor (NET) | 22 | 38.5 | 53.8 | 7.7 | 0.0 | 61.5 |
| Colorectal, neuroendocrine tumor (NET) | 10 | 70.0 | 30.0 | 0.0 | 0.0 | 30.0 |
| Ileum, neuroendocrine tumor (NET) | 49 | 76.6 | 23.4 | 0.0 | 0.0 | 23.4 |
| Lung, neuroendocrine tumor (NET) | 19 | 82.4 | 17.6 | 0.0 | 0.0 | 17.6 |
| Pancreas, neuroendocrine tumor (NET) | 102 | 52.1 | 40.6 | 5.2 | 2.1 | 47.9 |
| Pancreas, neuroendocrine carcinoma (NEC) | 11 | 45.5 | 0.0 | 27.3 | 27.3 | 54.5 |
| Gallbladder, neuroendocrine carcinoma (NEC) | 4 | 25.0 | 0.0 | 75.0 | 0.0 | 75.0 |
| Pancreas, neuroendocrine carcinoma (NEC) | 13 | 27.3 | 36.4 | 18.2 | 18.2 | 72.7 |
| Tumors of hematopoietic and lymphoid tissues | | | | | | |
| Hodgkin Lymphoma | 103 | 64.9 | 23.4 | 10.4 | 1.3 | 35.1 |
| Diffuse large B cell lymphoma (DLBCL) | 114 | 63.6 | 29.1 | 5.5 | 1.8 | 36.4 |
| Follicular lymphoma | 88 | 58.3 | 41.7 | 0.0 | 0.0 | 41.7 |
| T-cell Non-Hodgkin Lymphoma | 24 | 58.3 | 33.3 | 8.0 | 0.0 | 41.7 |
| Mantle cell lymphoma | 18 | 83.3 | 16.7 | 0.0 | 0.0 | 16.7 |
| Marginal zone lymphoma | 16 | 80.0 | 20.0 | 0.0 | 0.0 | 20.0 |
| Diffuse large B-cell lymphoma (DLBCL) in the testis | 16 | 80.0 | 13.3 | 6.7 | 0.0 | 20.0 |
| Burkitt lymphoma | 5 | 75.0 | 25.0 | 0.0 | 0.0 | 25.0 |
| Tumors of soft tissue and bone | | | | | | |
| Tenosynovial giant cell tumor | 45 | 9.3 | 88.4 | 2.3 | 0.0 | 90.7 |
| Angiomyolipoma | 91 | 95.2 | 4.8 | 0.0 | 0.0 | 4.8 |
| Angiosarcoma | 73 | 47.1 | 41.2 | 9.8 | 2.0 | 52.9 |
| Dermatofibrosarcoma protuberans | 21 | 25.0 | 68.8 | 6.3 | 0.0 | 75.0 |
| Ganglioneuroma | 14 | 90.0 | 0.0 | 0.0 | 0.0 | 10.0 |
| Granular cell tumor | 23 | 38.1 | 57.1 | 4.8 | 0.0 | 61.9 |
| Kaposi sarcoma | 8 | 66.7 | 33.3 | 0.0 | 0.0 | 33.3 |
| Leiomyoma | 50 | 57.5 | 42.5 | 0.0 | 0.0 | 42.5 |
| Leiomyosarcoma | 87 | 26.0 | 31.2 | 22.1 | 20.8 | 74.0 |
| Liposarcoma | 132 | 11.1 | 22.2 | 10.1 | 56.6 | 88.9 |
| Malignant peripheral nerve sheath tumor (MPNST) | 13 | 83.3 | 8.3 | 8.3 | 0.0 | 16.7 |
| Myofibrosarcoma | 26 | 52.0 | 8.0 | 4.0 | 36.0 | 48.0 |
| Neurofibroma | 117 | 64.9 | 24.7 | 7.8 | 2.6 | 35.1 |
| Sarcoma, not otherwise specified (NOS) | 75 | 38.2 | 25.0 | 8.8 | 27.9 | 61.8 |
| Paranglioma | 41 | 77.8 | 22.2 | 0.0 | 0.0 | 22.2 |
| Primitive neuroectodermal tumor (PNET) | 23 | 35.0 | 50.0 | 10.0 | 5.0 | 65.0 |
| Rhabdomyosarcoma | 7 | 28.6 | 0.0 | 0.0 | 71.4 | 71.4 |
| Schwannoma | 121 | 12.4 | 47.4 | 23.7 | 16.3 | 87.6 |
| Synovial sarcoma | 12 | 45.5 | 36.4 | 18.2 | 0.0 | 54.5 |
| Osteosarcoma | 39 | 87.5 | 12.5 | 0.0 | 0.0 | 12.5 |
| Chondrosarcoma | 43 | 41.4 | 6.9 | 10.3 | 41.4 | 58.6 |

https://doi.org/10.1371/journal.pone.0262877.t001
Fig 3. Ranking order of p16 immunostaining in human tumors. Both the frequency of positive cases (blue dots) and the frequency of strongly positive cases (orange dots) is shown.

https://doi.org/10.1371/journal.pone.0262877.g003
### Table 2. p16 immunostaining and tumor phenotype.

|                          | Analyzable n | p16 Immunostaining | p Value |
|--------------------------|--------------|--------------------|---------|
|                          |              | negative (%)       | weak (%) | moderate (%) | strong (%) |         |
| **Vulvar carcinoma**     |              |                    |         |             |           |         |
| All cancers              | 128          | 57.0               | 6.3     | 12.5        | 24.2       | 0.0880  |
| PT1                     | 43           | 51.2               | 2.3     | 18.6        | 27.9       |         |
| PT2                     | 67           | 58.2               | 7.5     | 11.9        | 22.4       |         |
| PT3-4                   | 14           | 78.6               | 14.3    | 0.0         | 7.1        |         |
| G1                      | 16           | 68.8               | 12.5    | 18.8        | 0.0        | 0.1567  |
| G2                      | 62           | 54.8               | 6.5     | 11.3        | 27.4       |         |
| G3                      | 30           | 56.7               | 6.7     | 13.3        | 23.3       |         |
| pN0                     | 68           | 50.0               | 4.4     | 16.2        | 29.4       | 0.1448  |
| pN+                     | 37           | 64.9               | 10.8    | 8.1         | 16.2       |         |
| **Endometrioid endometrial carcinoma** | | | | | | |
| All cancers              | 149          | 22.1               | 66.4    | 10.1        | 1.3        |         |
| PT1                     | 94           | 21.3               | 67.0    | 9.6         | 2.1        | 0.9198  |
| PT2                     | 23           | 21.7               | 65.2    | 13.0        | 0.0        |         |
| PT3-4                   | 29           | 20.7               | 69.0    | 10.3        | 0.0        |         |
| pN0                     | 7            | 15.6               | 75.6    | 6.7         | 2.2        | 0.4994  |
| pN+                     | 5            | 23.8               | 57.1    | 14.3        | 4.8        |         |
| **Serous high grade ovarian carcinoma** | | | | | | |
| All cancers              | 295          | 15.2               | 12.9    | 49.2        | 22.7       |         |
| PT1                     | 20           | 5.0                | 0.0     | 70.0        | 25.0       | 0.1266  |
| PT2                     | 38           | 18.4               | 13.2    | 42.1        | 26.3       |         |
| PT3                     | 208          | 15.9               | 14.4    | 47.1        | 22.6       |         |
| pN0                     | 65           | 15.4               | 9.2     | 53.9        | 21.5       | 0.1883  |
| pN1                     | 138          | 10.9               | 18.8    | 44.2        | 26.1       |         |
| **Invasive breast carcinoma of no special type** | | | | | | |
| All cancers              | 910          | 61.1               | 30.2    | 3.0         | 5.7        |         |
| PT1                     | 452          | 61.5               | 32.7    | 1.8         | 4.0        | 0.0738  |
| PT2                     | 358          | 62.8               | 25.7    | 3.6         | 7.8        |         |
| PT3-4                   | 70           | 61.4               | 28.6    | 2.9         | 7.1        |         |
| G1                      | 127.0        | 76.4               | 22.8    | 0.8         | 0.0        | <0.0001 |
| G2                      | 433.0        | 63.5               | 33.7    | 1.6         | 1.2        |         |
| G3                      | 349.0        | 52.7               | 28.4    | 5.4         | 13.5       |         |
| pN0                     | 468.0        | 65.5               | 27.4    | 3.1         | 1.4        | 0.8944  |
| pN+                     | 343.0        | 60.7               | 30.6    | 2.2         | 6.6        |         |
| **Urinary bladder carcinoma** | | | | | | |
| All cancers              | 1245         | 45.7               | 32.5    | 8.4         | 13.4       |         |
| pTa G2 low               | 145          | 19.3               | 80.0    | 0.7         | 0.0        | <0.0001 |
| pTa G2 high              | 121          | 51.2               | 43.0    | 2.5         | 3.3        |         |
| pTaG3                    | 138          | 26.8               | 48.6    | 13.8        | 10.9       |         |
| PT2 G3                   | 780          | 51.3               | 20.3    | 10.0        | 18.5       |         |
| Normal urothelium        | 24           | 91.7               | 8.3     | 0.0         | 0.0        | 0.0465  |
| Dysplasia                | 12           | 53.3               | 33.3    | 8.3         | 0.0        |         |
| **Clear cell renal cell carcinoma** | | | | | | |
| All cancers              | 620          | 98.9               | 1.0     | 0.1         | 0.0        |         |
| PT1                     | 365          | 98.9               | 1.1     | 0.0         | 0.0        | 0.4494  |
| PT2                     | 63           | 100.0              | 0.0     | 0.0         | 0.0        |         |
| PT3-4                   | 187          | 98.4               | 1.1     | 0.5         | 0.0        | 0.0      |
| ISUP 1                   | 192          | 99.5               | 0.5     | 0.0         | 0.0        | 0.7094  |
| ISUP 2                   | 204          | 99.0               | 1.0     | 0.0         | 0.0        |         |
| ISUP 3                   | 177          | 98.3               | 1.1     | 0.6         | 0.0        |         |
| ISUP 4                   | 38           | 97.4               | 2.6     | 0.0         | 0.0        |         |
| pN0                     | 98           | 99.0               | 1.0     | 0.0         | 0.0        | 0.5927  |
| pN≥1                     | 15           | 100.0              | 0.0     | 0.0         | 0.0        |         |

(Continued)
known inverse relationship of p53 alterations (a well-established cause for p16 upregulation) and MSI in colorectal cancer. Moreover, it has been shown that microsatellite instability leads to increased methylation of the p16 gene which may lead to reduced p16 expression or at least hinder p16 upregulation [37]. The results of our study do not exclude that p16 expression can also be reduced in some cancers as described in previous studies [15,38–41]. However, an entirely different experimental approach than the one selected for this study, with a much higher antibody concentration and a more sensitive staining protocol would be required to demonstrate reduced expression.

That a minimum of one case with at least a moderate p16 positivity was found in 100 of our 124 (80.6%) analyzed cancer types demonstrates that p16 immunostaining offers only limited...
Fig 4. p16 immunostaining and overall survival in patients with invasive breast cancer of no special type and urothelial carcinoma (pT2-4; G3). *The numbers do not add to the total number of tumors with clinical follow-up data, since only cases with evaluable p16 staining are included.

https://doi.org/10.1371/journal.pone.0262877.g004
support for defining a tumor’s site of origin. According to our data, there are only few occasions, where p16 immunostaining can assist in diagnosing the right tumor type. This applies for example—as previously suggested [42]—in the differentiation of high grade endometrial from serous carcinoma of the endometrium. In our study 40.6% of serous endometrial

Table 3. Association between p16 immunostaining and HPV status.

|                | p16 status | n   | HPV status |             |              |
|----------------|------------|-----|------------|-------------|--------------|
|                |            |     | negative   | positive    |              |
| All cancers    | negative   | 253 | 79.4       | 20.6        | <0.0001      |
|                | weak       | 54  | 74.1       | 25.9        |              |
|                | moderate   | 53  | 37.7       | 62.3        |              |
|                | strong     | 163 | 19.6       | 80.4        |              |
| Oral squamous cell carcinoma | negative | 55  | 89.1       | 10.9        | 0.0017       |
|                | weak       | 3   | 100.0      | 0.0         |              |
|                | moderate   | 4   | 100.0      | 0.0         |              |
|                | strong     | 9   | 33.3       | 66.7        |              |
| Squamous cell carcinoma of the pharynx | negative | 25  | 72.0       | 28.0        | <0.0001      |
|                | weak       | 4   | 25.0       | 75.0        |              |
|                | moderate   | 4   | 25.0       | 75.0        |              |
|                | strong     | 17  | 5.9        | 94.1        |              |
| Squamous cell carcinoma of the larynx | negative | 47  | 83.0       | 17.0        | 0.3388       |
|                | weak       | 2   | 100.0      | 0.0         |              |
|                | moderate   | 2   | 50.0       | 50.0        |              |
|                | strong     | 4   | 100.0      | 0.0         |              |
| Squamous cell carcinoma of the cervix | negative | 4   | 100.0      | 0.0         | <0.0001      |
|                | weak       | 2   | 0.0        | 100.0       |              |
|                | moderate   | 12  | 0.0        | 100.0       |              |
|                | strong     | 57  | 7.0        | 93.0        |              |
| Squamous cell carcinoma of the vagina | negative | 16  | 68.8       | 31.3        | 0.0077       |
|                | weak       | 2   | 100.0      | 0.0         |              |
|                | moderate   | 4   | 0.0        | 100.0       |              |
|                | strong     | 7   | 28.6       | 71.4        |              |
| Squamous cell carcinoma of the vulva | negative | 44  | 72.7       | 27.3        | 0.0571       |
|                | weak       | 12  | 83.3       | 16.7        |              |
|                | moderate   | 6   | 33.3       | 66.7        |              |
|                | strong     | 13  | 46.2       | 53.8        |              |
| Squamous cell carcinoma of penis | negative | 34  | 67.6       | 32.4        | <0.0001      |
|                | weak       | 6   | 16.7       | 83.3        |              |
|                | moderate   | 3   | 0.0        | 100.0       |              |
|                | strong     | 28  | 14.3       | 85.7        |              |
| Squamous cell carcinoma of the skin | negative | 19  | 100.0      | 0.0         | 0.0951       |
|                | weak       | 9   | 100.0      | 0.0         |              |
|                | moderate   | 2   | 50.0       | 50.0        |              |
|                | strong     | 6   | 100.0      | 0.0         |              |
| Squamous cell carcinoma of the anal canal | negative | 6   | 50.0       | 50.0        | 0.0723       |
|                | weak       | 2   | 0.0        | 100.0       |              |
|                | moderate   | 5   | 0.0        | 100.0       |              |
|                | strong     | 22  | 9.1        | 90.9        |              |

https://doi.org/10.1371/journal.pone.0262877.t003
carcinoma showed strong p16 immunostaining in comparison to 5.1% strong p16 positivity in endometroid endometrial carcinoma.

In normal tissues, moderate to high p16 immunostaining was only consistently seen in islets of Langerhans of the pancreas and in the pituitary gland. p16 immunostaining was largely absent in tissues which are prone to develop cancer such as urothelium and squamous cell epithelium of various sites. Given the high rate of p16 overexpression in various cancers developing from p16 negative cells, p16 immunostaining may serve as a parameter that might indicate malignancy in some organs. Based on our findings that might for example apply for liposarcoma (56.6% strong positive, vs negative in normal fat), leiomyosarcoma (21% strong positive, vs 0% strong positive cases in leiomyoma) or urothelial dysplasia (46.7% positive, vs negative in normal urothelium). The high utility of immunohistochemical p16 analysis in assessing cervical biopsies is based on the fact that almost all neoplasias in this location are due to HPV infection [43]. The imperfect correlation of p16 expression and HPV infection found in our analysis of 497 squamous cell carcinomas of different origins suggest low reliability for using p16 immunostaining as a surrogate for HPV infection in other cancer types, however.

Of note, in our study HPV could be detected in 20.6% of cases with absent p16 immunostaining. On the other hand, it is not surprising, that moderate to strong p16 positivity was found in 25–100% (average 30.7%) of HPV negative extra-genital squamous cell carcinomas, given the interaction of p16 with several important pathways. That aberrant p16 expression was not only seen in endothelial cells of a few cancers, but also in rare instances in endothelial cells in non-neoplastic tissue and in fibroblasts of the tumor stroma demonstrates, that substantial p16 upregulation can occasionally also occur in non-neoplastic tissue proliferation.

Our highly standardized analysis of 11,759 tumors from 124 different tumor entities enabled us to clarify the relative importance of p16 expression across tumor entities and to generate a ranking list according to the p16 positivity rate (Fig 3). It is of note, that many of the top ranked p16 positive tumor entities such as small cell neuroendocrine carcinoma of the bladder, Merkel cell carcinoma, small cell carcinoma of the lung and small cell neuroendocrine carcinoma of the prostate exhibit neuroendocrine differentiation. This observation is in line with data from an earlier study showing, that loss of Rb function, which can cause overexpression of p16, leads to neuroendocrine hypercellularity in the lung [44]. Although we are unaware of a specific role of p16 in neuroendocrine cellular functions, it is conspicuous that our normal tissue analysis had also identified the highest expression in neuroendocrine/endocrine cells of islets of Langerhans in the pancreas, and the adenohypophysis. Moreover, it is possible that the scattered p16 positive cells in the gastrointestinal tract also represent endocrine cells. Several other studies have also shown p16 overexpression in various neuroendocrine neoplasms of different origin [45–47].

More than 3000 studies have previously analyzed p16 expression in tumors by immunohistochemistry. The summary of the results of 448 of these studies in Fig 5 demonstrates, that highly discrepant data on the prevalence of p16 positivity exist for many tumor entities. This wide range of published p16 positivity rates makes it difficult to assess the potential significance of p16 immunohistochemistry in individual tumor entities and may also be responsible for conflicting data on the potential prognostic and diagnostic relevance of p16 expression in such tumor entities. That our own analyses of associations between clinico-pathological parameters of cancer aggressiveness and p16 expression mostly revealed only weak or even no associations seems to suggest, that p16 overexpression is not a feature that is dramatically linked to lethal cancer cell properties.

In summary, these results provide a comprehensive overview on p16 expression in human normal tissues and cancers. The absence of a significant p16 expression in most normal tissues in combination with a high frequency of p16 overexpression in cancers of all types demonstrates a significant role of p16 in cancer biology and suggest a general utility of p16 immunohistochemistry
as a potential aid to diagnose malignancy. The lack of striking associations of p16 immunostaining with clinico-pathological parameters for cancer aggressiveness in most analyzed cancer types argues against a major prognostic impact of p16 protein expression, however.

Supporting information

S1 Table. List of studies used to generate Fig 5.
(XLSX)

S2 Table. p16 positive and p16 negative normal tissues and associated tumor types.
(XLSX)

Acknowledgments

We are grateful to Melanie Witt, Inge Brandt, Sünje Seekamp, Maren Eisenberg, Gabriele Rieck, Sina Dietrich, Jana Hagemann and Tessa-Ann Saggau
for excellent technical support.

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