

Prevalence of S-methyl-5'-thioadenosine Phosphorylase (MTAP) Deficiency in Human Cancer

A Tissue Microarray Study on 13,067 Tumors From 149 Different Tumor Types

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Abstract: Loss of S-methyl-5'-thioadenosine phosphorylase (MTAP) expression is a common event in cancer leading to a critical vulnerability of cancer cells towards anti-cancer drugs. Homozygous *MTAP* deletions result in a complete expression loss that can be detected by immunohistochemistry (IHC). In this study, a tissue microarray containing 17,078 samples from 149 different tumor entities was analyzed by IHC, and complete MTAP loss was validated by fluorescence in situ hybridization. MTAP loss was observed in 83 of 149 tumor categories, including neuroendocrine neoplasms (up to 80%), Hodgkin lymphoma (50.0%), mesothelioma (32.0% to 36.8%), gastro-intestinal adenocarcinoma (4.0% to 40.5%), urothelial neoplasms (10.5% to 36.7%), squamous cell carcinomas (up to 38%), and various types of sarcomas (up to 20%) and non-Hodgkin lymphomas (up to 14%). Homozygous MTAP deletion was found in 90% to 100% of cases with MTAP expression

loss in most tumor categories. However, neuroendocrine tumors, Hodgkin lymphomas, and other lymphomas lacked MTAP deletions. MTAP deficiency was significantly linked to unfavorable tumor phenotype in selected tumor entities and the presence of PD-L1 expression on tumor cells, absence of PD-L1 expression on immune cells, and a low density of CD8⁺ lymphocytes. In summary, MTAP deficiency can occur in various tumor entities and is linked to unfavorable tumor phenotype and noninflamed tumor microenvironment, but is not always related to deletions. MTAP IHC is of considerable diagnostic value for the detection of neoplastic transformation in multiple different applications.

Key Words: MTAP, tissue micro array, immunohistochemistry, FISH, diagnostic marker

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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. N.G., N.W., R.S., M.K., G.S., and L.B.: conception, design, data collection, data analysis, and manuscript writing. N.W., M.L., S.D.R., S.K., V.R., F.V., F.L., C.F., A.M.L., A.M., R. Schlichter, T.K., A.H., E.B., S.S., A.H.M., P.L., D.D., S.M., F.J., T.S.C., T.H., F.G. U., and C.B.: pathology data analysis, data interpretation, and collection of samples. N.G., R. Simon, M.K., and C.H.M.: data analysis. N.G., R.S., G.S., and M.K.: study supervision.

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The enzyme S-methyl-5'-thioadenosine phosphorylase (MTAP) is essential for the salvage pathway of adenine synthesis.¹ The gene is located at 9p21.3, only 30 kb apart from the *CDKN2A* gene, which is deleted in up to 15% of cancers.²⁻⁴ Codeletions of *MTAP* occur in 80% to 90% of tumors with *CDKN2A* homozygous deletion.⁵ Evidence is accumulating that MTAP deficiency results in a critical vulnerability of cancer cells towards drugs targeting several different pathways.⁶ As adenine synthesis can only be maintained by de novo biosynthesis in MTAP deficient cancer cells, inhibition of enzymes for folate synthesis leads to increased cell death in experimental models and also showed significant anti-cancer efficiency in patients with urinary bladder cancer.⁶ MTAP deficient cells can also be targeted by drugs inhibiting protein arginine N-methyltransferase 5 (PRMT5) and methionine adenosyltransferase II, alpha (summarized in reference⁷). PRMT5 is an essential enzyme for the methylation of numerous proteins, thus regulating their activity,⁸ whereas MAT2A is essential for the synthesis of S-adenosylmethionine, the methyl donor, and substrate of PRMT5.^{9,10} Upregulation of PRMT5 was found to result in progression and poor prognosis in a variety of malignancies including breast cancer,¹¹ glioblastoma,¹² prostate cancer,¹³ pancreatic cancer,¹⁴ bladder cancer,¹⁵ leukemia,¹⁶ lymphoma,¹⁷ ovarian cancer,¹⁸ lung cancer,¹⁹ and gastric cancer.²⁰ Because PRMT5 is partially inhibited by the intracellular accumulation of the unprocessed MTAP metabolite MTA,²¹ MTAP deficiency makes cells more susceptible to drugs targeting PRMT5 or methionine adenosyltransferase II, alpha (summarized in reference⁷). A clinical phase I trial published in November 2023 has shown significant size reduction in tumor lesions of patients with *MTAP*-deleted cancers, including epithelioid malignant mesothelioma, non-small-cell lung cancer, malignant melanoma, and adenocarcinoma with PRMT5 inhibitor MRTX1719.²² Several other clinical trials targeting PRMT5 are ongoing.²³ Also of interest, both MTAP deficiency²⁴ and *CDKN2A* loss²⁵ could be linked to poor response to immune checkpoint inhibitor (CPI) therapy in urothelial carcinoma. 9p21 loss was also associated with poor response to CPI in lung cancer, melanoma, and miscellaneous solid tumors.²⁶

Considering these promising data and the abundance of 9p21 deletions in many cancer types, it is likely that many cancers from different entities may benefit from drugs targeting MTAP deficiency. Immunohistochemical detection of MTAP may represent a suitable surrogate for the detection of homozygous 9p21 deletions because MTAP is rather ubiquitously expressed in normal tissues, and its expression is completely lost in homozygously deleted cells. Because of frequent homozygous 9p21 deletions in pleural mesothelioma, MTAP immunohistochemistry (IHC) is used as a tool for supporting this diagnosis in histologic and cytologic specimens.²⁷ Only few studies have investigated MTAP expression by IHC in other tumor entities and described complete expression loss to occur in subsets of high-grade gliomas,²⁸ pulmonary adenocarcinoma,²⁹ esophageal adenocarcinomas,³⁰ serous borderline tumors and serous

carcinomas of the ovary,³¹ pancreatic intraepithelial neoplasia,³² Ewing sarcoma,³³ and osteosarcoma.³⁴ However, most previous studies on non-mesothelial neoplasms employed rather small patient cohorts, and many tumor entities have so far not been analyzed.

To comprehensively determine the prevalence of MTAP protein expression loss in cancer and to assess the potential diagnostic utility of MTAP IHC, we analyzed a preexisting set of tissue microarrays (TMAs) containing more than 17,000 tumor tissue samples from 149 different tumor types and subtypes, as well as 76 non-neoplastic tissue categories for MTAP expression by IHC in this study. All cancers with complete MTAP loss were also validated for *MTAP* deletions by fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

Tissue Microarrays

Our normal TMA was composed of 8 samples from 8 different donors from each of 76 different normal tissue types (608 samples on one slide). The cancer TMAs included a total of 17,078 primary tumors from 149 different tumor types and subtypes.

Detailed histopathologic and molecular data were available for cancers of the liver (n = 231), pancreas (n = 598), breast (n = 1208), neuroendocrine tumors (NETs; n = 138), and squamous cell carcinomas (SQCC) of various origin (n = 902). The composition of normal and cancer TMAs is described in the results section. All samples were 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 Fuerth, Germany. Tissues were fixed in 4% buffered formalin and then embedded in paraffin. The TMA manufacturing process was described earlier in detail.^{35,36} In brief, one tissue spot (diameter: 0.6 mm) per patient was used. The use of archived remnants of diagnostic tissues for TMA manufacturing, their analysis for research purposes, and the use of patient data were according to local laws (HmbKHG, §12), and the analysis had been approved by the local ethics committee (Ethics Commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration. Data on PD-L1 and CD8⁺ lymphocyte density were available for subsets of our tumors from a previous study.³⁷

Fluorescence In Situ Hybridization

Five micrometer TMA sections were deparaffinized with xylol, rehydrated through a graded alcohol series, and exposed to heat-induced denaturation for 10 minutes in a water bath at 99 °C in P1 pretreatment solution (Agilent Technologies; #K5799). For proteolytic treatment, slides were added to VP2000 protease buffer (Abbott; #2J.0730) for 200 minutes at 37 °C in a water bath. A commercial 315 kilobases (kb) FISH probe spanning the 194 kb region on chromosome 9p21 where the *CDKN2A* (27 kb) and the *MTAP* (135 kb) gene localize

was utilized for 9p21 copy number detection (ZytoLight SPEC CDKN2A/CEN 9 Dual Color Probe, Zytovision; #Z-2063). Loss of the 9p21 probe signal in FISH experiments indicates the presence of large deletions, including both CDKN2A and MTAP. Hybridization was performed overnight at 37°C in a humidified chamber. Posthybridization washes were done according to the manufacturer's direction at 37°C (Agilent Technologies; #K5799). Nuclei were counterstained with 125 ng/mL 4',6-diamino-2-phenylindole in antifade solution (Biozol; #VEC-H-1200). Stained tissues were manually interpreted with an epifluorescence microscope, and copy numbers of 9p21 and centromere 9 were estimated for each tissue spot. The presence of equal numbers of 9p21 and centromere 9 signals in tumor cell nuclei was considered as 9p21 normal. The presence of fewer 9p21 signals than centromere 9 probe signals in at least 60% of tumor cell nuclei or one 9p21 and one centromere 9 signal (monosomy of chromosome 9) in nearly all tumor cell nuclei were considered as heterozygous deletion. The complete absence of 9p21 signals but the presence of centromere 9 signals in the tumor cell nuclei and the presence of unequivocal 9p21 and centromere 9 signals in tumor-adjacent normal cell nuclei were considered homozygous 9p21 deletion.

Tissue spots lacking any detectable 9p21 signals in all (tumor and normal cell nuclei) or lack of any normal cells as an internal control for successful hybridization of the 9p21 probe were excluded from the analysis. These thresholds were based on the results of a previous study in which 100% concordance in *PTEN* copy number status was achieved using FISH and single nucleotide polymorphism array hybridization in a cohort of 72 prostate cancers.³⁸ Representative FISH findings are shown in Supplemental Figure 1 (Supplemental Digital Content 1, <http://links.lww.com/PAS/B920>).

Immunohistochemistry

A TMA containing bladder carcinomas with 9p21 wild type (n = 20), heterozygous deletions (n = 20), and homozygous deletions (n = 20) was used to titrate the anti-MTAP antibodies to obtain maximal staining in non-neoplastic cells while background staining was still lacking in homozygously deleted cancers (data not shown). The optimal protocol was as follows. Freshly prepared 2.5 µm TMA sections were applied on 1 day in 1 experiment in a Dako Omnis automated stainer (Agilent Technologies) using the EnVision FLEX, High pH Kit (Agilent Technologies, #GV800). Slides were deparaffinized with Clearify agent (Agilent Technologies, #GC810) and exposed to heat-induced antigen retrieval for 30 minutes at 97°C in target retrieval solution, high pH reagent (part of Agilent kit #GV800). Primary antibody specific for MTAP (recombinant rabbit monoclonal, MSVA-741R, MS Validated Antibodies GmbH, #5293-741R) was applied at ambient temperature for 30 minutes at a dilution of 1:50. Endogenous peroxidase activity was blocked with peroxidase blocking-reagent (part of Agilent kit #GV800) for 3 minutes. Bound antibody was visualized using the EnVision FLEX, High pH kit reagents DAB+ Chromogen

and Substrate Buffer (parts of Agilent kit #GV800) and EnVision FLEX + Rabbit LINKER (Agilent Technologies; #GV809) according to the manufacturer's directions. The sections were counterstained with hemalaun. For tumor tissues, the average staining intensity of unequivocally neoplastic cells was estimated as 0, 1+, 2+, and 3+. For the classification of a tumor as completely negative (0, complete loss of MTAP), the presence of unequivocal MTAP staining in tumor adjacent normal tissue was required. Tumors with a complete absence of MTAP staining in cancerous cells and a lack of stromal cells with unequivocal MTAP staining were considered "non-informative." For the purpose of antibody validation, the normal tissue TMA was also analyzed by the mouse monoclonal MTAP antibody 2G4 (Abnova; # H00004507-M01) with a manual protocol. These 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 7.8 tris-EDTA-citrate puffer. Endogenous peroxidase activity was blocked with Dako Peroxidase Blocking Solution (Agilent Technologies; #S2023) for 10 minutes. The MTAP antibody 2G4 was applied at 37°C for 60 minutes at a dilution of 1:40. The sections were counterstained with hemalaun.

Statistics

Statistical calculations were performed with JMP17 software (SAS). Contingency tables and the χ^2 test were performed to search for associations between MTAP immunostaining and tumor phenotype and PD-L1 immunostaining. Analysis of variance analysis was used to search for associations between MTAP immunostaining and the density of CD8⁺ lymphocytes (tumor micro-environment).

RESULTS

Technical Issues

A total of 13,067 (76.5%) of 17,078 tumor samples were interpretable in our tumor TMA analysis. Non-interpretable samples demonstrated a lack of unequivocal tumor cells, loss of the entire tissue spot, or a complete absence of MTAP staining in both tumor cells and adjacent stroma cells. Sufficient numbers of samples (≥ 4) of each normal tissue type were evaluable.

MTAP in Normal Tissue

MTAP immunostaining revealed a nuclear and/or cytoplasmic positivity at variable intensity of most cell types. Particularly strong staining occurred in the ovarian stroma, endothelial cells, inflammatory cells, cytotrophoblast and chorion cells of the placenta, urothelium, epithelial cells of the epididymis and the seminal vesicles, breast glands, salivary glands, spermatogonia, adrenal gland, thyroid, and the adenohypophysis. MTAP staining was particularly low or even absent in hepatocytes, heart muscle, fat tissue, and the brain. Representative images are shown in Figure 1. All these findings were obtained by using the recombinant rabbit monoclonal antibody

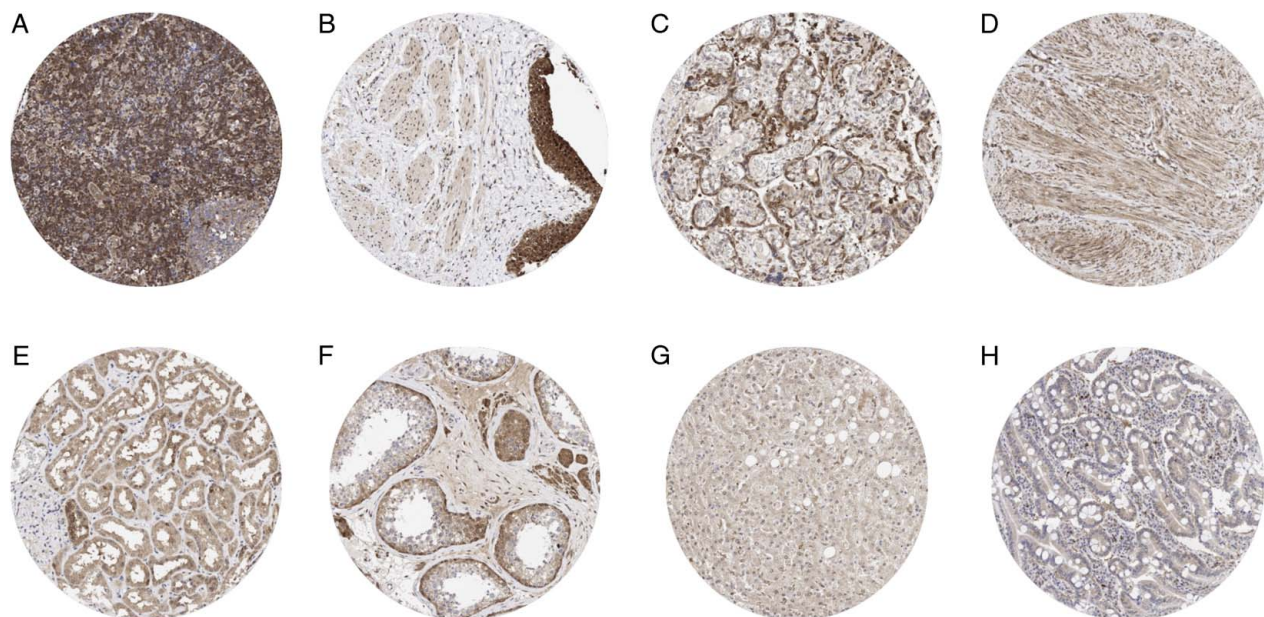


FIGURE 1. MTAP immunostaining of normal tissues. The panels show MTAP immunostaining of variable intensity for almost every cell type in each tissue. MTAP staining was particularly intense in interfollicular lymphocytes of a lymph node (A), urothelial cells in the urinary bladder (B), and in cytotrophoblast cells of the placenta (C). A moderate to strong MTAP staining was also seen in myometrium cells of the uterus (D), tubular cells of the kidney (E), and in spermatogonia and Leydig cells of the testis (F). MTAP staining was only weak in maturing spermatids of the testis (F), hepatocytes of the liver (G), and in colorectal epithelial cells (H).

MSVA-741R and the mouse monoclonal 2G4 antibody (Supplemental Fig. 2, Supplemental Digital Content 2, <http://links.lww.com/PAS/B921>) and, therefore, considered to be specific. By using the antibody 2G4, a strong additional staining of goblet cells of the entire intestinal mucosa was observed. This staining was considered an antibody-specific cross-reactivity of antibody 2G4.

MTAP in Cancer

MTAP immunostaining in tumors was of various intensities and included a variable mixture of cytoplasmic and nuclear staining. MTAP immunostaining was considered weak (1+) in 6,339 (48.5%), moderate in 4,802 (36.7%), and strong in 993 (7.6%) of tumors. A complete loss (0) of MTAP staining was seen in 933 (7.1%) of tumors. At least one case with a complete MTAP expression loss was observed in 83 (55.7%) of 149 tumor categories (Table 1). Representative images of MTAP deficient tumors lacking 9p21 deletions and harboring 9p21 deletions are shown in Figures 2 and 3. MTAP staining was most commonly lost in Hodgkin lymphoma (50%), neuroendocrine neoplasms of various sites (up to 80%), mesothelioma (32.0% to 36.8%), biliopancreatic adenocarcinomas (17.9% to 40.5%), urothelial neoplasms (10.5% to 36.7%), malignant melanoma (15.7% to 28.4%), mucinous carcinoma of the ovary (29.2%), SQCC of various organs of origin (up to 38%), various types of sarcomas (up to 20%), various types of non-Hodgkin lymphoma (up to 14%), adenocarcinoma of the lung (12.0%), and in gastric adenocarcinoma (4.0% to 11.9%).

FISH Validation

FISH validation was performed for all cancers with complete MTAP loss by IHC. It showed that the concordance rate between MTAP IHC and FISH varied greatly between tumor entities (Fig. 4). The concordance rate was 100% in mesothelioma, urothelial carcinoma and SQCC of the urinary bladder, adenocarcinoma and SQCC of the esophagus, malignant peripheral nerve sheath tumor, sarcoma not otherwise specified, as well as in several other entities with low numbers of tumors analyzed. It was 90% to 99% in many other tumor types, including gallbladder adenocarcinoma, mucinous carcinoma of the ovary, malignant melanoma (including nodal metastases), SQCC of the oral cavity, and hepatocellular carcinoma (HCC). Most notably, there were various other tumor entities with common MTAP loss by IHC but an almost complete lack of concomitant 9p21 deletions. These included Hodgkin lymphoma, several other lymphoma types, as well as NETs and carcinomas of various sites of origin. A graphical representation of the ranking order of cancers with MTAP deficiency by IHC and their 9p21 status obtained by FISH is given in Figure 4. If neuroendocrine and hematological neoplasms were excluded, a homozygous 9p21 deletion occurred in 96.7% of the tumors with a complete MTAP expression loss.

MTAP Deficiency and Tumor Phenotype

The relationship between MTAP deficiency and clinically important histopathologic and molecular tumor features in carcinomas from different sites is shown in

TABLE 1. MTAP Immunostaining in Human Tumors

Tumor category	Tumor entity	on TMA (n)	MTAP immunostaining				
			Analyzable (n)	0 (%)	1+ (%)	2+ (%)	3+ (%)
Tumors of the skin	Basal cell carcinoma of the skin	89	68	2.9	45.6	38.2	13.2
	Benign nevus	29	23	0.0	4.3	26.1	69.6
	Squamous cell carcinoma of the skin	145	103	9.7	68.9	19.4	1.9
	Malignant melanoma	65	51	15.7	19.6	49.0	15.7
	Malignant melanoma lymph node metastasis	86	67	28.4	32.8	23.9	14.9
	Merkel cell carcinoma	2	1	0.0	100.0	0.0	0.0
Tumors of the head and neck	Squamous cell carcinoma of the larynx	109	81	24.7	56.8	18.5	0.0
	Squamous cell carcinoma of the pharynx	60	59	11.9	28.8	49.2	10.2
	Oral squamous cell carcinoma (floor of the mouth)	130	119	11.8	44.5	37.8	5.9
	Pleomorphic adenoma of the parotid gland	50	38	0.0	60.5	36.8	2.6
	Warthin tumor of the parotid gland	104	63	0.0	60.3	39.7	0.0
	Adenocarcinoma, NOS (papillary cystadenocarcinoma)	14	6	0.0	100.0	0.0	0.0
	Salivary duct carcinoma	15	3	66.7	33.3	0.0	0.0
	Acinic cell carcinoma of the salivary gland	181	53	1.9	62.3	35.8	0.0
	Adenocarcinoma NOS of the salivary gland	109	29	10.3	65.5	20.7	3.4
	Adenoid cystic carcinoma of the salivary gland	180	33	6.1	27.3	63.6	3.0
	Basal cell adenocarcinoma of the salivary gland	25	12	0.0	33.3	41.7	25.0
	Basal cell adenoma of the salivary gland	101	26	0.0	26.9	65.4	7.7
	Epithelial-myoepithelial carcinoma of the salivary gland	53	18	0.0	44.4	55.6	0.0
	Mucoepidermoid carcinoma of the salivary gland	343	149	4.0	65.8	29.5	0.7
	Myoepithelial carcinoma of the salivary gland	21	10	0.0	70.0	30.0	0.0
	Myoepithelioma of the salivary gland	11	8	0.0	37.5	62.5	0.0
	Oncocytic carcinoma of the salivary gland	12	2	0.0	100.0	0.0	0.0
	Polymorphous adenocarcinoma, low grade, of the salivary gland	41	2	0.0	0.0	50.0	50.0
	Pleomorphic adenoma of the salivary gland	53	22	4.5	45.5	40.9	9.1
	Tumors of the lung, pleura and thymus	Adenocarcinoma of the lung	196	167	12.0	64.7	21.6
Squamous cell carcinoma of the lung		80	59	16.9	62.7	20.3	0.0
Mesothelioma, epithelioid		40	25	32.0	24.0	40.0	4.0
Mesothelioma, biphasic		29	19	36.8	42.1	15.8	5.3
Thymoma		29	21	0.0	52.4	47.6	0.0
Lung, NET		29	28	53.6	46.4	0.0	0.0
Squamous cell carcinoma of the vagina		78	54	18.5	38.9	37.0	5.6
Tumors of the female genital tract	Squamous cell carcinoma of the vulva	157	119	21.0	48.7	27.7	2.5
	Squamous cell carcinoma of the cervix	136	119	1.7	57.1	35.3	5.9
	Adenocarcinoma of the cervix	23	22	4.5	31.8	59.1	4.5
	Endometrioid endometrial carcinoma	338	296	0.7	61.5	35.5	2.4
	Endometrial serous carcinoma	86	67	1.5	38.8	41.8	17.9
	Carcinosarcoma of the uterus	57	52	0.0	59.6	34.6	5.8
	Endometrial carcinoma, high grade, G3	13	12	0.0	16.7	75.0	8.3
	Endometrial clear cell carcinoma	9	7	0.0	85.7	14.3	0.0
	Endometrioid carcinoma of the ovary	130	96	1.0	39.6	44.8	14.6
	Serous carcinoma of the ovary	580	443	1.1	38.1	47.9	12.9
	Mucinous carcinoma of the ovary	101	65	29.2	44.6	18.5	7.7
	Clear cell carcinoma of the ovary	51	32	6.3	62.5	31.3	0.0
	Carcinosarcoma of the ovary	47	40	7.5	40.0	42.5	10.0
	Granulosa cell tumor of the ovary	44	42	2.4	38.1	45.2	14.3
	Leydig cell tumor of the ovary	4	4	0.0	0.0	100.0	0.0
	Sertoli cell tumor of the ovary	1	1	0.0	0.0	100.0	0.0
	Sertoli Leydig cell tumor of the ovary	3	3	0.0	33.3	66.7	0.0
	Steroid cell tumor of the ovary	3	3	0.0	0.0	100.0	0.0
	Brenner tumor	41	39	5.1	12.8	66.7	15.4
	Tumors of the breast	Invasive breast carcinoma of no special type	499	454	1.5	48.0	40.5
Lobular carcinoma of the breast		192	166	0.0	34.3	54.8	10.8
Medullary carcinoma of the breast		23	19	5.3	21.1	42.1	31.6
Tubular carcinoma of the breast		20	11	0.0	27.3	54.5	18.2
Mucinous carcinoma of the breast		29	26	0.0	26.9	65.4	7.7
Phyllodes tumor of the breast		50	47	0.0	27.7	57.4	14.9
Tumors of the digestive system	Adenomatous polyp, low-grade dysplasia	50	42	0.0	33.3	64.3	2.4
	Adenomatous polyp, high-grade dysplasia	50	48	0.0	27.1	60.4	12.5
	Adenocarcinoma of the colon	2483	1892	0.8	37.3	41.8	20.1

TABLE 1. (continued)

Tumor category	Tumor entity	on TMA (n)	MTAP immunostaining				
			Analyzable (n)	0 (%)	1+ (%)	2+ (%)	3+ (%)
	Gastric adenocarcinoma, diffuse type	215	151	4.0	64.9	31.1	0.0
	Gastric adenocarcinoma, intestinal type	215	168	11.9	44.0	38.7	5.4
	Gastric adenocarcinoma, mixed type	62	57	5.3	57.9	29.8	7.0
	Adenocarcinoma of the esophagus	83	45	13.3	46.7	28.9	11.1
	Squamous cell carcinoma of the esophagus	76	37	27.0	32.4	37.8	2.7
	Squamous cell carcinoma of the anal canal	91	78	5.1	43.6	46.2	5.1
	Cholangiocarcinoma	58	43	16.3	51.2	32.6	0.0
	Gallbladder adenocarcinoma	51	42	40.5	42.9	16.7	0.0
	Gallbladder Klatskin tumor	42	34	11.8	61.8	17.6	8.8
	HCC	312	294	9.2	60.9	24.8	5.1
	Ductal adenocarcinoma of the pancreas	659	506	32.4	42.1	22.3	3.2
	Pancreatic/ampullary adenocarcinoma	98	78	17.9	51.3	29.5	1.3
	Acinar cell carcinoma of the pancreas	18	16	18.8	50.0	18.8	12.5
	GIST	62	55	3.6	56.4	38.2	1.8
	Appendix, NET	25	14	35.7	42.9	21.4	0.0
	Colorectal, NET	12	10	70.0	10.0	20.0	0.0
	Ileum, NET	53	47	78.7	19.1	2.1	0.0
	Pancreas, NET	101	89	11.2	71.9	12.4	4.5
	Colorectal, NEC	14	13	7.7	38.5	46.2	7.7
	Ileum, NEC	8	5	80.0	0.0	20.0	0.0
	Gallbladder, NEC	4	4	0.0	25.0	75.0	0.0
	Pancreas, NEC	14	12	8.3	58.3	33.3	0.0
Tumors of the urinary system	Noninvasive papillary urothelial carcinoma, pTa G2 low-grade	87	76	10.5	27.6	25.0	36.8
	Noninvasive papillary urothelial carcinoma, pTa G2 high-grade	80	66	28.8	16.7	45.5	9.1
	Noninvasive papillary urothelial carcinoma, pTa G3	126	101	18.8	26.7	42.6	11.9
	Urothelial carcinoma, pT2-4 G3	735	573	29.0	30.4	28.8	11.9
	Squamous cell carcinoma of the bladder	22	21	38.1	52.4	4.8	4.8
	Small cell NEC of the bladder	5	4	0.0	0.0	50.0	50.0
	Sarcomatoid urothelial carcinoma	25	12	8.3	33.3	50.0	8.3
	Urothelial carcinoma of the kidney pelvis	62	49	36.7	26.5	28.6	8.2
	Clear cell renal cell carcinoma	1287	1109	0.6	62.2	36.4	0.7
	Papillary renal cell carcinoma	368	318	1.3	45.3	47.5	6.0
	Clear cell (tubulo) papillary renal cell carcinoma	26	21	0.0	52.4	42.9	4.8
	Chromophobe renal cell carcinoma	170	146	0.0	73.3	26.0	0.7
	Oncocytoma of the kidney	257	222	0.0	49.5	49.5	0.9
Tumors of the male genital organs	Adenocarcinoma of the prostate, Gleason 3+3	83	67	0.0	47.8	43.3	9.0
	Adenocarcinoma of the prostate, Gleason 4+4	80	65	0.0	32.3	60.0	7.7
	Adenocarcinoma of the prostate, Gleason 5+5	85	70	0.0	38.6	57.1	4.3
	Adenocarcinoma of the prostate (recurrence)	258	159	1.9	42.8	49.1	6.3
	Small cell NEC of the prostate	2	1	0.0	100.0	0.0	0.0
	Seminoma	682	454	0.4	72.5	24.2	2.9
	Embryonal carcinoma of the testis	54	44	0.0	40.9	50.0	9.1
	Leydig cell tumor of the testis	31	31	0.0	22.6	77.4	0.0
	Sertoli cell tumor of the testis	2	2	0.0	50.0	50.0	0.0
	Sex cord stromal tumor of the testis	1	1	0.0	100.0	0.0	0.0
	Spermatocytic tumor of the testis	1	1	0.0	0.0	100.0	0.0
	Yolk sac tumor	53	40	0.0	62.5	30.0	7.5
	Teratoma	53	40	0.0	45.0	55.0	0.0
	Squamous cell carcinoma of the penis	92	86	2.3	64.0	25.6	8.1
Tumors of endocrine organs	Adenoma of the thyroid gland	113	99	0.0	58.6	39.4	2.0
	Papillary thyroid carcinoma	391	335	0.0	41.2	57.3	1.5
	Follicular thyroid carcinoma	154	104	0.0	55.8	42.3	1.9
	Medullary thyroid carcinoma	111	74	5.4	83.8	10.8	0.0
	Parathyroid gland adenoma	43	37	0.0	35.1	56.8	8.1
	Anaplastic thyroid carcinoma	45	39	5.1	43.6	48.7	2.6
	Adrenal cortical adenoma	48	46	0.0	41.3	58.7	0.0
	Adrenal cortical carcinoma	27	27	0.0	59.3	33.3	7.4
	Pheochromocytoma	51	44	2.3	86.4	11.4	0.0
	Paraganglioma	41	36	0.0	72.2	27.8	0.0
Tumors of hematopoietic and	Hodgkin lymphoma	103	50	50.0	46.0	4.0	0.0

TABLE 1. (continued)

Tumor category	Tumor entity	on TMA (n)	MTAP immunostaining				
			Analyzable (n)	0 (%)	1+ (%)	2+ (%)	3+ (%)
lymphoid tissues							
	B-SLL, B-cell type (B-CLL)	50	48	0.0	39.6	41.7	18.8
	DLBCL	113	108	4.6	56.5	33.3	5.6
	Follicular lymphoma	88	86	7.0	60.5	31.4	1.2
	T-cell non-Hodgkin lymphoma	25	22	13.6	50.0	36.4	0.0
	Mantle cell lymphoma	18	18	0.0	33.3	61.1	5.6
	Marginal zone lymphoma	16	14	14.3	35.7	28.6	21.4
	DLBCL in the testis	16	14	7.1	85.7	7.1	0.0
	Burkitt lymphoma	5	4	0.0	50.0	50.0	0.0
Tumors of soft tissue and bone							
	Granular cell tumor	23	15	0.0	0.0	93.3	6.7
	Leiomyoma	50	41	0.0	90.2	9.8	0.0
	Leiomyosarcoma	94	70	5.7	57.1	37.1	0.0
	Atypical lipomatous tumor/well-differentiated liposarcoma	18	11	0.0	54.5	45.5	0.0
	Dedifferentiated liposarcoma	54	42	0.0	73.8	26.2	0.0
	Myxoid liposarcoma	18	14	0.0	64.3	35.7	0.0
	Pleomorphic liposarcoma	3	1	0.0	100.0	0.0	0.0
	Pleomorphic rhabdomyosarcoma	3	1	0	100	0	0
	MPNST	15	13	30.8	53.8	15.4	0.0
	Myofibrosarcoma	26	24	0.0	41.7	54.2	4.2
	Angiosarcoma	42	26	0.0	50.0	46.2	3.8
	Angiomyolipoma	91	60	0.0	85.0	15.0	0.0
	Dermatofibrosarcoma protuberans	21	16	6.3	50.0	43.8	0.0
	Ganglioneuroma	14	12	0.0	91.7	8.3	0.0
	Kaposi sarcoma	8	4	0.0	100.0	0.0	0.0
	Neurofibroma	117	104	0.0	90.4	9.6	0.0
	Sarcoma, NOS	74	63	14.3	46.0	39.7	0.0
	Ewing sarcoma	23	10	0.0	50.0	30.0	20.0
	Alveolar rhabdomyosarcoma	2	1	0.0	100.0	0.0	0.0
	Pleomorphic rhabdomyosarcoma	2	2	50.0	50.0	0.0	0.0
	Embryonal rhabdomyosarcoma	3	2	0.0	0.0	50.0	50.0
	Schwannoma	122	113	0.9	59.3	38.1	1.8
	Synovial sarcoma	12	10	0.0	10.0	30.0	60.0
	Osteosarcoma	19	12	0.0	66.7	25.0	8.3
	Chondrosarcoma	15	10	20.0	40.0	40.0	0.0
	Rhabdoid tumor	5	5	0.0	40.0	60.0	0.0
	Solitary fibrous tumor	17	17	0.0	41.2	52.9	5.9

Immunostaining score “0” indicates complete loss of MTAP expression.

CLL indicates chronic lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; GIST, gastrointestinal stromal tumor; MPNST, malignant peripheral nerve sheath tumor; NEC, neuroendocrine carcinoma; NOS, not otherwise specified; SLL, small lymphocytic lymphoma.

Table 2. MTAP deficiency was linked to the presence of nodal metastasis ($P = 0.0349$) and high grade ($P = 0.0156$) in HCC, advanced pT stage in NETs ($P = 0.0166$), as well as high grade ($P = 0.0034$), advanced pT stage ($P = 0.0299$) and absence of human papillomavirus (HPV) infection ($P < 0.0001$) in SQCC of different organs of origin. Markedly higher rates of MTAP deficiency in SQCC lacking HPV infection were also observed in most subgroups of tumors from individual organs, although statistical significance was only reached for SQCC of the cervix ($P = 0.0028$) and the anal canal ($P = 0.045$; Table 3). The frequency of MTAP deficiency further increased with pT and grade in invasive breast cancer of no special type and in pancreatic adenocarcinoma, although not all these differences reached statistical significance. Across all tumor entities, tumors with a complete MTAP loss were more often PD-L1 positive ($P = 0.0125$), had fewer PD-L1 positive macrophages ($P =$

0.0008), and fewer cytotoxic ($CD8^+$) lymphocytes in their microenvironment ($P = 0.0042$; Table 4).

DISCUSSION

The highly standardized analysis of 13,067 tumors from 149 different tumor entities provided an overview of the prevalence of “MTAP deficiency” across different tumor entities and identified Hodgkin lymphoma, neuroendocrine neoplasms of various sites of origin, mesothelioma, biliarypancreatic adenocarcinoma, urothelial carcinoma, malignant melanoma, mucinous carcinoma of the ovary, and SQCC from various organs as the most commonly MTAP negative tumors. For most of these tumor entities, 9p21 deletions have previously been described, but the reported prevalence often varied greatly due to the use of different methods, and the rate of homozygous deletions was not described in most of these

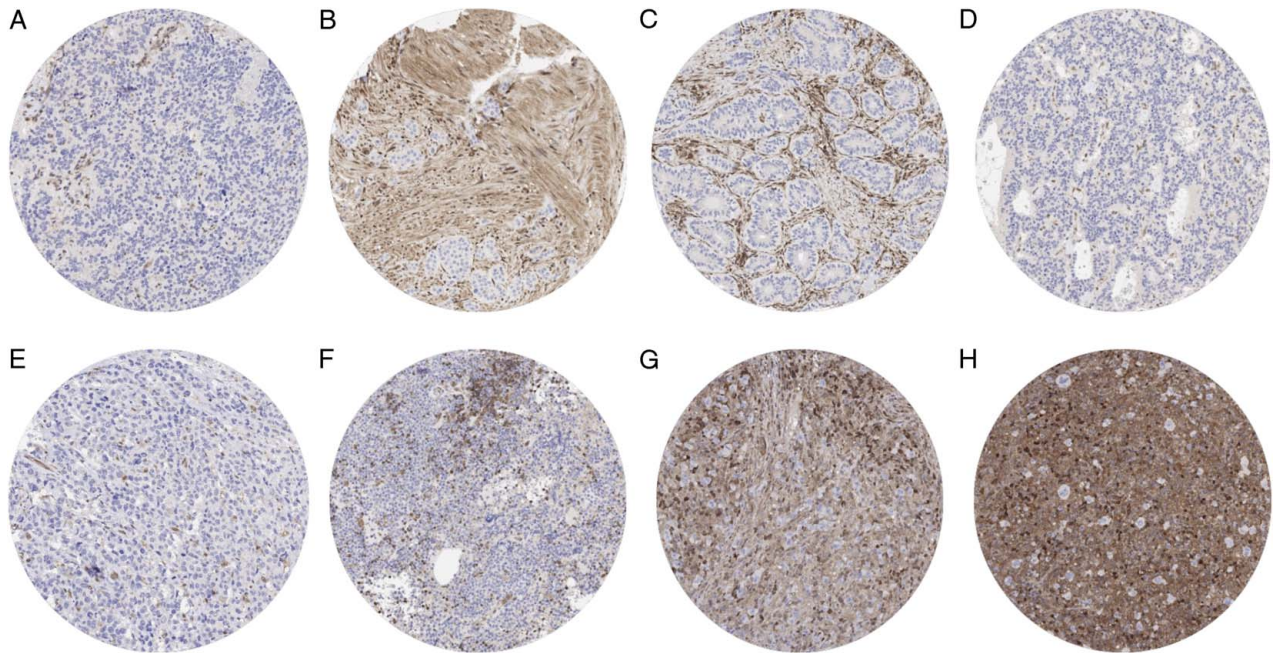


FIGURE 2. MTAP loss in tumors lacking 9p21 deletion. MTAP staining was completely absent in tumor cells of a NET of the lung (A), a NET of the ileum (B), a colorectal NET (C), a pancreatic NET (D), a T-cell non-Hodgkin lymphoma (E), a marginal cell lymphoma (F) and in 2 cases of Hodgkin lymphoma (G and H). Distinct MTAP positivity of intermingled stroma cells serves as a positive internal control.

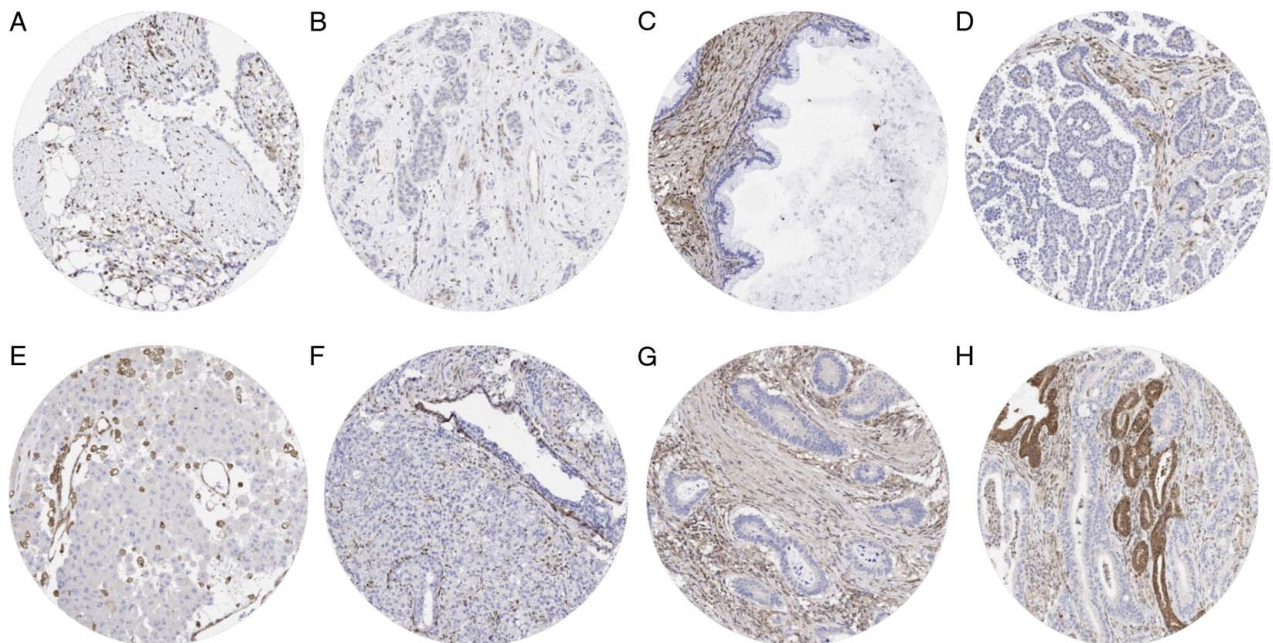


FIGURE 3. MTAP loss in tumors with homozygous 9p21 deletion. MTAP staining was completely lacking in tumor cells of an (epithelioid) malignant mesothelioma (A), a urothelial carcinoma of the renal pelvis (B), a mucinous ovarian carcinoma (C), a serous high-grade ovarian carcinoma (D), a malignant melanoma (E), a recurrent adenocarcinoma of the prostate (F), a colorectal adenocarcinoma (G), and a ductal adenocarcinoma of the pancreas (H). Distinct MTAP positivity of intermingled stroma cells and of non-neoplastic epithelial cells serves as a positive internal control.

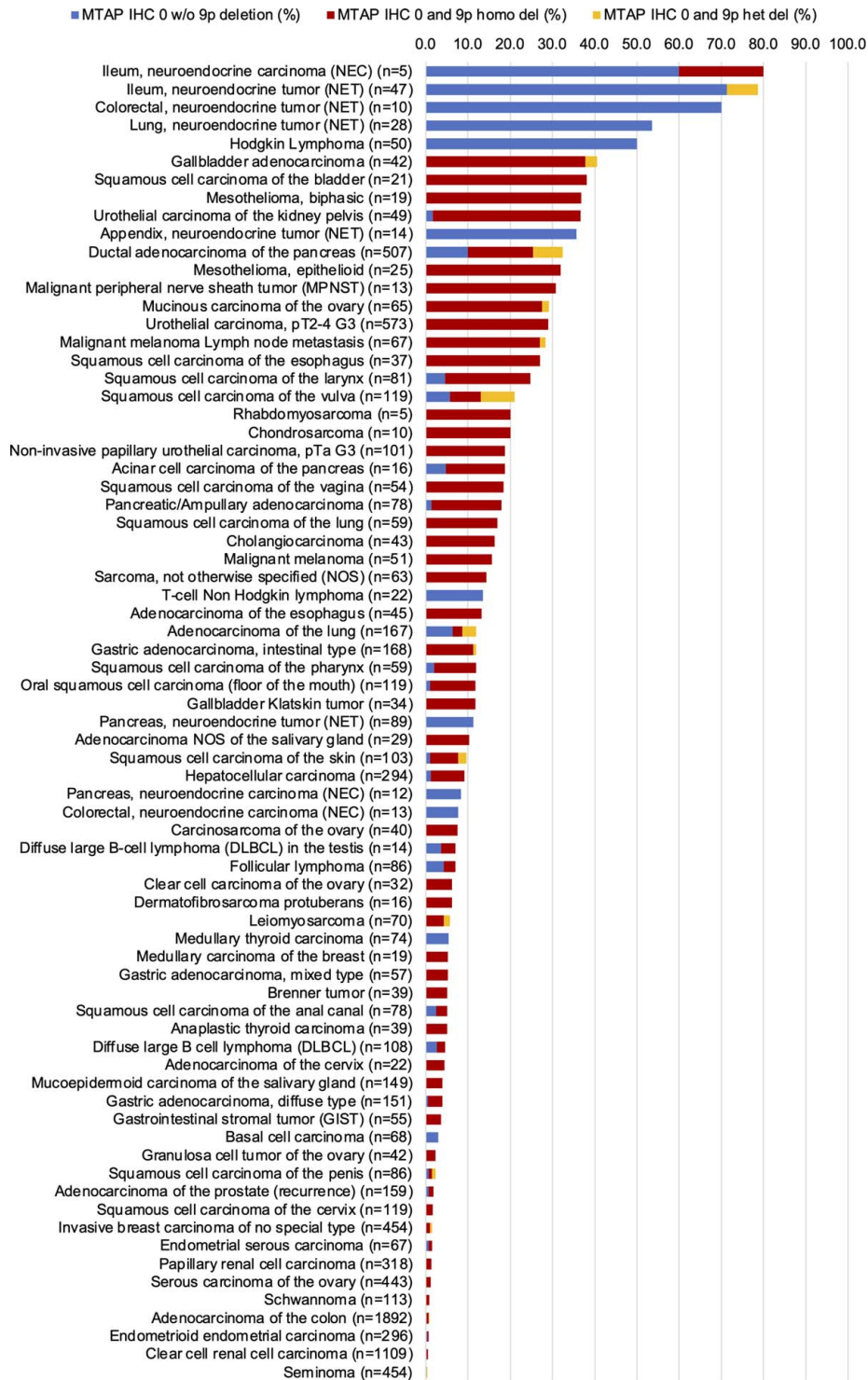


FIGURE 4. Concordance rate of MTAP IHC and 9p21 copy number analysis.

studies. For example, the prevalence of 9p21 deletions ranged at least from 25% to 88% in malignant mesothelioma,³⁹⁻⁴¹ from 18% to 70% in pancreatic adenocarcinoma,⁴²⁻⁴⁴ from 14% to 96% in urothelial carcinoma^{45,46} , from 16% to 84% (6% to 41% homo-

zygous) in malignant melanoma,⁴⁷⁻⁵¹ from 25% to 100% (14% to 18% homozygous) in ovarian cancer,⁵²⁻⁵⁴ from 28% to 68% in HCC,^{55,56} and from 6% to 18% in prostatic adenocarcinoma.^{57,58}

In a recent study on more than 2,600 urothelial neo-

TABLE 2. MTAP Immunostaining and Tumor Phenotype

Entity	Pathologic parameters	n	MTAP status (%) [*]		P	
			Loss	Retained		
HCC	Tumor stage	pT1	77	6.5	93.5	0.0911
		pT2	83	10.8	89.2	—
		pT3-pT4	59	18.6	81.4	—
	Nodal stage	pN0	74	10.8	89.2	0.0349
		pN+	42	26.2	73.8	—
	Grade	G1	36	0.0	100.0	0.0156
G2		128	10.9	89.1	—	
G3		52	13.5	86.5	—	
NETs [†]	Tumor stage	pT1	28	35.7	64.3	0.0166
		pT2	24	37.5	62.5	—
		pT3	36	47.2	52.8	—
	Nodal stage	pT4	32	71.9	28.1	—
		pN0	45	37.8	62.2	0.0946
	pN+	59	54.2	45.8	—	
Pancreatic adenocarcinoma	Tumor stage	pT1	7	14.3	85.7	0.6582
		pT2	59	28.8	71.2	—
		pT3	316	29.7	70.2	—
		pT4	24	37.5	62.5	—
	Nodal stage	pN0	81	33.3	66.7	0.4091
		pN+	325	28.6	71.4	—
	Grade	G1	16	18.8	81.3	0.3964
		G2	282	31.2	68.8	—
		G3	88	26.1	73.9	—
Invasive breast cancer of no special type	Tumor stage	pT1	162	0.0	100.0	0.1019
		pT2	194	1.5	98.5	—
	Nodal stage	pT3-pT4	35	2.9	97.1	—
		pN0	198	0.5	99.5	0.8765
	Grade	pN+	98	0.6	99.4	—
		G1	13	0.0	100.0	0.0268
		G2	221	0.0	100.0	—
		G3	161	2.5	97.5	—
SQCCs [‡]	Tumor stage	pT1	235	10.6	89.4	0.0299
		pT2	250	10.0	90.0	—
		pT3	121	19.0	81.0	—
		pT4	125	17.6	82.4	—
	Nodal stage	pN0	276	12.0	88.0	0.0615
		pN+	285	17.5	82.5	—
	Grade	G1	30	0.0	100.0	0.0034
		G2	341	15.0	85.0	—
		G3	234	9.8	90.2	—
	HPV	Negative	275	18.2	81.8	<0.0001
Positive		233	5.6	94.4	—	

*MTAP loss: no MTAP immunostaining, MTAP retained: MTAP 1+ to 3+ immunostaining.

[†]Neuroendocrine tumors of the appendix, colorectum, ileum, pancreas, and lung.

[‡]Squamous cell carcinomas of the oral cavity (n = 119), pharynx (n = 59), larynx (n = 82), esophagus (n = 36), lung (n = 59), cervix (n = 1139), vagina (n = 54), vulva (n = 100), penis (n = 77), skin (n = 68), and the anal canal (n = 78).

HPV indicates human papillomavirus.

plasms, we found a very high sensitivity and specificity of our MTAP IHC assay for the detection of homozygous 9p21 deletions (Gorbokon et al, manuscript submitted). In this study, 98.4% of 252 urothelial tumors with a complete MTAP expression loss had a homozygous 9p21 deletion, and only 3.9% of 258 tumors with homozygous 9p21 deletions showed MTAP immunostaining. Other authors had also applied MTAP IHC to tumor cohorts with predefined 9p21 deletion status and found sensitivities between 59% and 100% and specificities between 96% and 100% for detection of homozygous deletions in mesothelioma, astrocytoma, and meningioma (Supplemental Table 1, Supplemental Digital Content 3, <http://links.lww.com/PAS/B922>). Our FISH validation of all MTAP IHC negative

cases revealed that such a near-complete FISH/IHC concordance does not apply to all tumor entities. Especially in neuroendocrine neoplasms, Hodgkin lymphomas, and in several other lymphoma entities, including T-cell lymphoma, loss of MTAP expression was never or only rarely due to a 9p21 deletion. Considering that within these tumor entities, a fraction of tumors also showed high-level MTAP expression and that normal precursor cells such as pancreatic islet cells or lymphocytes exhibited a distinct MTAP positivity, MTAP negativity in these tumors cannot be caused by particularly low MTAP levels in their cells of origin. In concordance with our data, Woollard et al⁵⁹ identified MTAP expression loss in a significant fraction of T-cell lymphomas that lacked 9p21 deletions. As a potential

TABLE 3. MTAP Immunostaining and HPV Status in SQCCs of Various Origins

Tumor entity	HPV status	n	MTAP status (%)*		P
			Loss	Retained	
Oral squamous cell carcinoma	Negative	66	15.2	84.8	0.0578
	Positive	12	0.0	100.0	—
Squamous cell carcinoma of the pharynx	Negative	22	13.6	86.4	0.8368
	Positive	34	11.8	88.2	—
Squamous cell carcinoma of the larynx	Negative	48	31.3	68.8	0.7177
	Positive	8	25.0	75.0	—
Squamous cell carcinoma of the cervix	Negative	9	22.2	77.8	0.0028
	Positive	67	0.0	100.0	—
Squamous cell carcinoma of the vagina	Negative	15	26.7	73.3	0.4638
	Positive	13	15.4	84.6	—
Squamous cell carcinoma of the vulva	Negative	45	24.4	75.6	0.4485
	Positive	24	16.7	83.3	—
Squamous cell carcinoma of the penis	Negative	29	3.4	96.6	0.7537
	Positive	45	2.2	97.8	—
Squamous cell carcinoma of the skin	Negative	36	8.3	91.7	0.6787
	Positive	1	0.0	100.0	—
Squamous cell carcinoma of the anal canal	Negative	5	20.0	80.0	0.0450
	Positive	29	0.0	100.0	—

*MTAP loss: no MTAP immunostaining, MTAP retained: MTAP 1+ to 3+ immunostaining.
HPV indicates human papillomavirus.

cause for MTAP downregulation, these authors observed MTAP promoter hypermethylation and subsequent MTAP downregulation in one T-cell lymphoma cell line. Hellerbrand et al⁶⁰ also found promoter hypermethylation to cause MTAP downregulation in HCC cell lines lacking genomic 9p21 deletions.

Although MTAP deficiency regularly occurs in the absence of 9p21 deletions in some tumor entities, we conclude from our data that homozygous *MTAP* deletion may represent the sole mechanism for MTAP deficiency in non-hematological and non-neuroendocrine cancers. Methodological issues may have caused many or even all of the discrepant cases in tumor entities with high rates of 9p21 deletions. As IHC and FISH analysis had not been executed on consecutive TMA sections, it appears likely that small tumor cell groups with unequivocal MTAP deficiency could not be confirmed by FISH in a fraction of cases because the respective cell population were not present on the respective FISH-stained sections. That small MTAP deficient tumor cell groups can easily be detected by IHC (Supplemental Fig. 3, Supplemental Digital Content 4, <http://links.lww.com/PAS/B923>) further

emphasized the high utility of MTAP IHC for the detection of MTAP deficiency.

Several studies had earlier suggested a treatment-agnostic impact of MTAP deficiency on the clinical course of cancer. For example, a relationship between MTAP deficiency/9p21 deletions and unfavorable tumor features has earlier been described in cohorts of 99 non-small-cell lung cancer,⁶¹ 75 oral SQCC,⁶² 35 head and neck SQCC,⁶³ 48 adenosquamous carcinoma of the pancreas,⁶⁴ 140 HCC,⁶⁵ 22 thymic carcinomas,⁶⁶ 146 gastrointestinal stromal tumors,⁶⁷ 113 Ewing sarcoma family of tumors,³³ and 62 bladder cancers.⁶⁸ In our own previous study on 636 patients undergoing cystectomy for muscle-invasive urothelial bladder cancer, both MTAP expression loss and 9p21 deletions were—independent of pT and pN stage—associated with poor outcome (Gorbokon et al, manuscript submitted). Significant associations between MTAP deficiency and unfavorable tumor features in HCC, NETs, and SQCC in this study are also in line with an increased aggressiveness of MTAP-deficient cancers.

A direct impact of MTAP deficiency on the anti-tumoral immune response has been suggested as a possible explanation for a prognostic role of 9p21 deletions and/or MTAP expression loss.⁶⁹ The significant correlation of MTAP deficiency with PD-L1 expression in tumor cells and a low density of CD8⁺ lymphocytes across all analyzed tumor entities is in line with this assumption, and also consistent with earlier reports describing a non-inflamed microenvironment and poor response to CPI in MTAP deficient/9p21 deleted cancers.^{26,70} The strong inverse correlation of MTAP deficiency with HPV infection in SQCC may suggest that MTAP deficiency is specifically not required in cancer cells having HPV-induced cancer-driving pathways activated. An inverse correlation between MTAP/9p21 loss and HPV infection has earlier been found in SQCC of the urinary bladder⁷¹ and in

TABLE 4. MTAP Immunostaining Versus PD-L1 Immunostaining and CD8⁺ T-cell Density

MTAP status*	n	PD-L1 positive (% of tumors)		CD8 ⁺ density (cells/mm ²)	
		Tumor cells	Immune cells	n	Mean ± SE
Loss	559	19.9	61.9	321	179.2 ± 28.2
Retained	7262	15.7	71.8	3915	263.3 ± 8.1
P	—	0.0125	0.0008	—	0.0042

*MTAP loss: no MTAP immunostaining, MTAP retained: MTAP 1+ to 3+ immunostaining.

oropharyngeal carcinomas.⁷²

Our data also suggest a considerable diagnostic utility of MTAP IHC in surgical pathology. The complete MTAP expression loss in 32.0% of our epithelioid and in 36.8% of the biphasic mesotheliomas supports the established role of MTAP IHC for the distinction of malignant mesothelioma from benign mesothelial conditions. Earlier studies had reported an MTAP loss in 12% to 77% of epithelioid mesotheliomas.^{73,74} It is assumed that homozygous 9p21 deletions do not occur in benign mesothelial proliferations.⁷⁵ Our data support several further potential diagnostic applications for MTAP IHC in the distinction of neoplastic from benign changes. In urothelial and skin samples, MTAP loss could support the diagnosis of urothelial dysplasia or melanoma. Both diagnoses are often difficult to establish based on morphology alone.^{76,77} More generally, a distinct MTAP loss in suspicious cells could support the diagnosis of a malignant process in small biopsies or cytologic specimens from various sites including the pancreas, bile ducts, soft tissues, the lung, or lymphatic tissues.

Given the potential clinical importance of MTAP expression analysis and the large scale of our study, emphasis was placed on a thorough validation of our assay. To assure the specificity of our immunostaining, our antibody was validated by a comparison of its staining pattern with a different independent antibody, as suggested by the International Working Group for Antibody Validation.⁷⁸ Our exceedingly large panel of 76 different normal tissues assures that virtually all proteins occurring in cells of adult humans were subjected to the cross-reactivity screening. That all nuclear and cytoplasmic staining patterns observed by MSVA-741R were also seen by 2G4 is strong evidence for the specificity of MSVA-741R. The additional staining of gastrointestinal mucus-producing goblet cells by 2G4, which was not observed by MSVA-741R, represents an antibody-specific cross-reactivity of 2G4 (Supplemental Fig. 2, Supplemental Digital Content 2, <http://links.lww.com/PAS/B921>). It cannot be excluded that this cross-reactivity may obscure MTAP expression loss in some mucus-producing tumors.

In summary, our data provide a comprehensive overview of the prevalence of MTAP deficiency in cancer and identify cancer entities that might most benefit from cancer drugs targeting MTAP deficiency. It will be interesting to study the potential intratumoral heterogeneity of MTAP loss in affected tumor types, as this could challenge the success of such therapies, and whether tumor entities with MTAP expression loss in the absence of deletion will equally well respond to these drugs. Our findings also highlight the diagnostic utility of MTAP IHC as a marker for neoplastic transformation in various different applications, including the distinction of mesothelioma, urothelial dysplasia, or melanoma from benign conditions.

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