AN IMMUNOHISTOCHEMICAL AND MOLECULAR GENETIC STUDY OF 60 COLORECTAL CARCINOMA BRAIN METASTASES IN PURSUIT OF PREDICTIVE BIOMARKERS FOR CANCER THERAPY.

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AN IMMUNOHISTOCHEMICAL AND MOLECULAR GENETIC STUDY OF 60 COLORECTAL CARCINOMA BRAIN METASTASES IN PURSUIT OF PREDICTIVE BIOMARKERS FOR CANCER THERAPY.

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Running title: Tumor treatment predictive biomarkers in CRC

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ABSTRACT

Colorectal carcinoma brain metastases (n=60) were studied using next-generation sequencing and immunohistochemistry. *RAS* and *BRAF* mutations were detected in 58.2% and 7.3% of cases, respectively. Patients with *RAS*- and *BRAF*-mutant tumors could potentially benefit from the treatment with inhibitors. *TP53* mutations were detected in 69.1% of metastases. Moreover, altered p53 expression was seen in 91.2% of cases. *APC* mutations were present in 41.8% of tumors. Diffuse nuclear accumulation of β-catenin was seen in 10.2% of metastases, although only 1 *CTNNB1* mutant was identified. Nevertheless, targeting p53 and Wnt/β-catenin pathways may have potential therapeutic implications. Casein kinase 1α1 expression indicating susceptibility to protein kinase inhibitors, was seen in 95% metastases including 10 with strong immunoreactivity. The immune checkpoint marker CD276, a promising target for immunotherapy, was present on tumor cells in 50.8% of metastases and on stromal cells in almost all cases. PRAME, another immunotherapy target, was expressed in 21.7% of tumors. HER2 membrane immunostaining detected in 13.3% of cases implicated potential treatment with HER2 inhibitors. Expression of SLFN11, a predictor of response to DNA-damaging chemotherapies, and a biomarker of sensitivity to PARP inhibitors was seen in 8.3% of tumors. In 6.7% of metastases loss or partial loss of MTAP expression suggested sensitivity to PRMT5 inhibitors. CD44v5 expressed in 35% of cases indicated potential therapeutic utility of anti-CD44v5 monoclonal antibody treatment. Identification of predictive biomarkers through genomic profiling and proteomic analyses is a crucial step toward individually tailored therapeutic regimens for patients with colorectal carcinoma brain metastases.

INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of cancer-associated death worldwide [1]. CRC can metastasize to any organ including the brain, although the overall average incidence of CRC brain metastases (BMs) is low ranging from 0.6 to 3.2% [2]. Patients with CRC BMs have poor outcomes with significant morbidity and mortality regardless of treatment that includes surgical resection, postoperative radiation, and chemotherapy [3]. The latter is often hampered by chemoresistance and a lack of drug delivery across the blood-brain barrier (BBB) [4,5]. Yet several highly promising delivery technologies to circumvent the BBB have been developed recently [5].

Progress in cancer genetics and immunology has laid foundation for the development of immuno- and targeted therapies. Combining molecularly targeted therapies with immune checkpoint inhibitors, conventional chemotherapy, or radiotherapy can synergistically inhibit multiple signaling pathways and reinforce anti-tumor effects of the treatment [6]. The identification of predictive biomarkers through genomic profiling and proteomic analyses is essential for optimal patient selection and rational design and optimization of combination regimens [7].

The aim of this study was to characterize a cohort of 60 CRC BMs using targeted next-generation sequencing (NGS) and immunohistochemistry (IHC) and identify predictive biomarkers for chemo-, molecularly targeted- and immuno- therapy.

MATERIAL AND METHODS

Formalin-fixed paraffin embedded (FFPE) samples of 60 de-identified CRC BMs were assembled in tissue microarrays (TMAs) as previously reported [8]. The histologic classification was done according to the “WHO Classification of Tumours of the Digestive System, 5th ed” [9].

Immunohistochemistry

Immunohistochemistry was performed using Ventana BenchMark Ultra (Ventana Medical Systems, Tucson, AZ) or Leica Bond-Max automated immunostainer (Leica Biosystems, Bannockburn, IL) and antibodies to the following antigens: Cytokeratin 20 (CK20), Caudal Type Homeobox 2 (CDX2), DNA-mismatch repair (MMR) proteins [MutL Homolog 1 (MLH1), PMS1 Homolog 2 (PMS2), MutS Homolog 2 (MSH2) and MutS Homolog 6 (MSH6)], β-catenin (CTNNB1), Tumor Protein P53 (p53), Erb-B2 Receptor Tyrosine Kinase 2 (HER2), Methylthioadenosine Phosphorylase (MTAP), Schlafen Family Member 11 (SLFN11), Casein kinase 1 alpha 1 (CK1 α 1), Cluster of differentiation (CD) 44 variant 5 (CD44v5), Programmed cell death protein 1 (PD-1/CD279), Programmed death-ligand 1 (PD-L1/CD274), B7 homolog 3 protein (B7-H3/CD276) and PReferentially expressed Antigen in MElanoma (PRAME). The percentage of positive cells was estimated for each case. Diffuse (d), patchy (p) and focal (f) immunostaining were defined, respectively, as ≥80, <80% ≥10 and <10 of positive tumor cells. Scattered positive cells were excluded from the focal category. Also, the intensity of immunostaining was estimated as strong (3), intermediate (2), and weak (1) in some cases. Predictive biomarkers such as HER2, MTAP, SLFN11, CK1 α 1, CD44v5, CD279, CD274, CD276, and PRAME were selected based on the literature review and availability of antibodies. Antibodies and immunohistochemical protocols are provided in Supplemental Table 1.

Targeted DNA next generation sequencing

Tumor DNA was extracted from FFPE samples using Maxwell® RSC DNA FFPE kit and a Maxwell® RSC instrument (Promega, Madison, WI). The Ion Torrent™ (Life Technologies/Thermo Fisher Scientific, Waltham, MA) next-generation sequencing platform and Ion AmpliSeq™ Cancer Hotspot Panel v2 Kit (targeting 50 commonly mutated oncogenes and tumor suppressor genes) were used for genotyping as previously described [8].

RESULTS

Demographic and clinicopathologic data

Demographic and clinicopathologic data are summarized in Table 1 and Supplemental Figure 1. CRC BMs (n=60) diagnosed in Caucasians of Europe were studied. The cohort included 25 females and 35 males with median age 67 and 65 years, respectively. The location of metastatic brain tumors was known in 45 cases. Thirteen BMs involved cerebellum, 11 frontal-, 6 temporal-, 5 parietal-, and 4 occipital- lobe. Three metastases involved frontoparietal, parietotemporal, or occipitotemporal regions. One tumor penetrated the frontoparietotemporal area. In 2 cases, dural metastases occurred. Primary tumor location was known in 37 cases. Six CRCs were from the right colon including 2 from caecum, and 1 of each from ascending colon, hepatic flexure, and transvers colon. The exact location of 1 right colon tumor was unknown. Thirty-one primary tumors were diagnosed in the left colon including 1 in descending, 6 in sigmoid, 1 in rectosigmoid junction and 18 in rectum. In 5 cases the exact location in the left colon was unknown. Most of CRC BMs were moderately (n=26) or poorly (n=32) differentiated adenocarcinomas. A well differentiated morphology was seen in 1 case. Two moderately differentiated CRCs focally displayed either mucinous or signet ring cell differentiation. One mucinous adenocarcinoma was diagnosed.

Immunohistochemistry

The results of IHC studies are summarized in Table 2. All but 1 BMs were CDX2-positive with a diffuse expression pattern seen in 54 cases. CK 20 was present in 53 tumors, although focal expression or scattered positive cells were noticed in 10 and 6 cases, respectively. Microsatellite instability was rare with loss of MLH1/PMS2 expression in 5% (3/60) of tumors. Expression of β-

catenin was evaluated in 59 BMs. Most of the tumors (n=49) revealed prominent membrane positivity although in 5 and 4 cases, respectively, patchy immunoreactivity or lack of staining was noticed. Nuclear accumulation occurred in 34 BMs. However, diffuse immunoreactivity was seen only in 6 cases. In the remaining 28 tumors β-catenin nuclear accumulation occurred either focally (n=14) or in scattered cells. p53 pathologic expression pattern was observed in 91.2% (52/57) of analyzed metastases. Forty-two tumors showed diffuse and strong p53 nuclear staining, while 10 cases were negative. CK1 α 1 expression either strong (Figure 2A) or moderate was seen, respectively, in 16.7% (10/60) and 41.7% (25/60) of BMs. The remaining 25 cases revealed weak CK1 α 1 IHC (n=22) or no staining (n=3). CD44v5 was expressed in 35% (21/60) CRC BMs (Figure 2B). In 3 cases expression pattern was diffuse, while 14 tumors showed either patchy (n=6) or focal positivity. HER2 IHC was positive in 10 tumors (Figure 2C). However, 2 tumors revealed only nuclear staining. BMs with membrane immunoreactivity (n=8) displayed either diffuse, strong (n=2) or intermediate (n=1) or patchy, weak positivity. SLFN11 expression was seen in 5 tumors of which 3 revealed strong and diffuse immunoreactivity (Figure 2D). Loss of cytoplasmic MTAP staining was noted in 4 cases, although focal in 2 tumors. Diffuse PRAME immunoreactivity was seen in 8 cases while 5 tumors revealed patchy staining (Figure 2E). None of 59 BMs expressed PD-1 or PD-L1 and only scattered positive tumor infiltrating immunocompetent cells were seen in 24 (40.7%) and 12 (20.3%) cases, respectively. However, 50.8% (30/59) of BMs revealed CD276 positivity, with focal expression pattern in 8 cases. Moreover, CD276 was prominently expressed in tumor stromal cells in 92.7% (51/55) of cases (Figure 2F). Some tumors expressed multiple predictive biomarkers. This is further highlighted in Supplemental Figure 1.

Targeted DNA NGS

DNA of sufficient quality was extracted from 55 metastatic tumors and examined by NGS. *RAS* was the most frequently mutated oncogene (58.2%, 32/55). There were 29 *KRAS* and 3 *NRAS* mutually exclusive mutations identified. Most of *KRAS* mutations were in codon 12 and 13 (n=23) with p.G12V substitution being the most common (n=10). *PIK3CA* mutations (n=10) with 3 exceptions coexisted with *KRAS* alterations. Most of these mutations (n=8) clustered in exon 9 hotspots p.E542, p.E545 and p.Q546. *BRAF* mutations including 3 p.V600E were identified in 4 (7.3%) tumors. The remaining 16 metastases except for 1 tumor with *ERBB2* mutation revealed no alteration in analyzed oncogenes, although harbored tumor suppressor gene mutations. *TP53* tumor suppressor gene was mutated in 69.1% (38/55), while *APC* and *CTNNB1*, core components of the canonical Wnt/β-catenin pathway, were mutated, respectively, in 41.8% (23/55) and 1.8% (1/55) of BMs. Mutations in other tumor suppressor genes including *ATM* (n=4), *FBXW7* (n=4), *PTEN* (n=5), *PTPN11*(n=1) and *SMAD4* (n=1) were identified in 13 metastases and except 2 cases were mutually exclusive. However, these alterations frequently coexisted with *KRAS*, *TP53*, and *APC* mutations. Tumor mutation profiles are illustrated in Supplemental Figure 1. Detailed NGS results are listed case-by-case in Supplemental Table 2.

DISCUSSION

Almost 60% of CRC BMs harbored *RAS* mutations. A similar frequency was previously reported [10]. Tumors driven by *RAS* tend to develop BMs more often than other primary colorectal adenocarcinomas [11]. For decades, *RAS* mutants were undruggable targets. However, discovery of covalent inhibitors targeting KRAS p.G12C offered possibility of targeted therapy [12,13]. Although, inhibitor monotherapies have not shown meaningful clinical impact in CRC patients, a combination of

KRAS p.G12C inhibitors with other therapies such as anti-epidermal growth factor receptor drugs or checkpoint inhibitors demonstrated promising efficacy in preclinical and clinical studies [14,15]. However, a low frequency of p.G12C mutation reported in current and other studies is a significant factor limiting success of KRAS p.G12C inhibitor targeted therapy [10]. Recently developed non-covalent pan-KRAS inhibitor which suppresses a broad range of KRAS mutants including all reported in this study, might be a breakthrough in the treatment of metastatic CRC driven by *KRAS* mutations [16]. A dual inhibition of MEK pathway and CDK4/6 demonstrated therapeutic efficacy in K-, and NRAS mutant patient-derived xenografts and a co-clinical trial [17].

Activation of the PI3K/AKT/mTOR pathway promotes CRC cell proliferation and survival [18]. Mutations in *PIK3CA* and *PTEN*, key components of the PI3K/AKT/mTOR pathway were identified in 18% and 9% of CRC BMs. Although several PI3K inhibitors have been developed and evaluated by preclinical studies and in clinical trials throughout the last decade, the efficacy of these therapeutics was limited due to the complex nature of the PI3K/AKT/mTOR pathway, which crosstalk with other pathways including RAS/RAF/MAPK and Wnt/β-catenin pathway [19].

Most CRCs driven by *BRAF* p.V600E belong to the consensus molecular subtype 1 characterized by distinctive features such as hypermutations, microsatellite instability, and immune activation [20]. Previous study reported *BRAF* p.V600E in 9% of CRC BMs [10]. In this investigation, 3 *BRAF* p.V600E mutants (5.5%) including 1 with multiple *APC*, *PTEN* and *TP53* mutations and deficient DNA mismatch repair (dMMR) were identified. *BRAF* p.V600E CRCs poorly respond to standard therapies [21]. However, recent trials showed that the combined BRAF and MEK inhibition and PD-1 immunotherapy augmented tumor response to the treatment [22].

TP53 mutations were detected in 69% of CRC BMs. Moreover, IHC revealed altered p53 expression in 91% of cases. Although *TP53* mutants have been considered “undruggable,” several

therapeutic strategies have been developed including degradation of mutant p53 and restoration of wild-type activity [23]. More recent preclinical experiments on *TP53* mutant colorectal and pancreatic cancer models revealed that *TP53* mutation status is a predictive biomarker for the treatment with combinations of trifluorothymidine and poly(ADP-ribose) polymerase inhibitors (PARPi) agents [24].

SLFN11 is a member of the SLFN family of genes implicated in important biological functions in mammals such as the control of cell proliferation and induction of immune responses [25]. Recently, SLFN11 expression status has emerged as a biomarker for the prediction of the response to conventional chemotherapy. Both in vitro studies using cell lines and patient-derived xenograft models, and clinical trials documented positive correlation between expression of SLFN11 and tumor cell sensitivity to DNA-damaging (DDAs) and PARPi agents [26,27]. In this study, 5 tumors including 2 with patchy positivity expressed SLFN11.

Reported frequency of HER2 positive brain metastases has varied from 12 to 21% [28-30]. In this study 13% of metastases revealed positive membrane staining, although most cases (5 of 8) were HER2-low tumors. The detection of HER2 low expression level is becoming increasingly important because of novel targeted agents, antibody drug conjugates, using HER2 as a docking site. A full blood-brain barrier-penetrant, highly selective HER2 inhibitor, DZD1516 was proven in pre-clinical and clinical studies to be effective in treatment of intracranial breast cancer metastases [31].

Dysregulation of the Wnt/β-catenin signaling pathway was implicated in tumorigenesis and progression of CRCs [32]. More than half of CRC BMs harbored either *APC* mutations or revealed nuclear accumulation of β-catenin, findings suggesting pathological signaling. Thus, targeting Wnt/β-catenin pathway with inhibitors, antagonists and agonists may have therapeutic value, although preclinical and clinical studies are still at an early stage [33,34].

Casein kinase 1 alpha 1 (CK1 α 1) encoded by *CSNK1A1* belongs to the CK1 protein family. This multifunctional protein has serine/threonine protein kinase activity and is one of the main components of the Wnt/β-catenin signaling pathway. CK1α was implicated in the development and progression of human cancer including CRC [35]. Over the past several years, a significant effort has been made to utilize protein kinase inhibitors in cancer treatment [36]. Epiblastin A, an adenosine triphosphate (ATP)-mediated competitive inhibitor of CK1α has been shown to inhibit cell-line-derived and patient-derived tumor xenograft CRC mice models [35]. The RNA interference and genome editing and immunotherapies targeting CK1 through the Wnt signaling pathway are among other potential therapeutic strategies [37]. The current study documented CK1 α 1 expression in 95% of CRC BMs with >50% showing intermediate to strong (17%) immunoreactivity. Thus, CK1 appears to be a potential therapeutic target in CRC BMs.

ATM loss of function mutations was reported in approximately 7% of colorectal carcinomas by The Cancer Genome Atlas Network (https://www.genome.gov). Although preclinical studies have shown that loss of ATM expression due to biallelic mutations sensitize human tumors to DNA-damaging chemotherapies, radiation, and DNA damage response inhibitors including ataxia telangiectasia and Rad3-related protein inhibitors, clinical trials have yielded mixed results [38]. In this cohort of CRC BMs, only 4 tumors (7%) harbored non-biallelic ATM mutations. Thus, clinical exploitation of this genetic deficiency remains elusive.

Deletion of the chromosome 9p21 (Chr9p21) locus involving *CDKN2A*, which encodes p19-ARF and p16-INK4a tumor suppressors, occurs in approximately 15% of human cancers. Chr9p21 deletion frequently extends proximal to *CDKN2A* causing co-deletion of the 5′-methylthioadenosine phosphorylase (*MTAP*) gene [39]. *MTAP* encodes an enzyme required for the metabolism of polyamines and purines, which plays a key role in the purine/methionine salvage pathway [40]. In

cancer cells, MTAP deficiency is impaired by depletion of the protein arginine methyltransferase 5 (PRMT5) because of the accumulation of methylthioadenosine (MTA). Physiologically, MTAP cleaves MTA to generate precursor substrates for methionine and adenine salvage pathways [40]. Several therapeutic strategies for the treatment of MTAP-deficient tumors have been developed. More recently, MRTX1719 (Mirati Therapeutics, San Diego, CA), the MTA-cooperative PRMT5 inhibitor that selectively binds the PRMT5-MTA complex has been shown to inhibit tumor growth in cancer cell lines and tumor xenograft models. Moreover, MRTX1719 is undergoing clinical trial (NCT0524550) in patients with unresectable or metastatic solid tumors harboring MTAP deletion [41,42]. In this study, MTAP expression was fully or partially lost in a small fraction (4/60, 7%) of CRC BMs. Nevertheless, an inhibition of the PRMT5-MTA complex could be a therapeutic option in such cases.

Immunotherapy is considered a promising treatment strategy for solid tumors including CRC [43]. Clinical CRC trials confirmed durable antitumor benefit of pembrolizumab in dMMR metastatic CRCs including a patient with brain metastasis [44,45]. In the current cohort, the incidence of proficient DNA mismatch repair (pMMR) and dMMR tumors corresponded to the previously published frequency in metastatic CRCs [46]. As reported in pMMR CRCs, frequency of tumor-infiltrating immune cells expressing PD-1 or PD-L1 was low suggesting limited benefit from the treatment targeting PD-1/PD-L1 axis [47].

Prominent expression of the CD276, also known as B7 homolog 3 (B7-H3) immunoregulatory protein, has been reported in many human malignancies. Because of restricted expression in normal tissues, the B7-H3 immune checkpoint molecule has become a target for therapeutic interventions and several promising strategies have been developed including a new class of antineoplastic agents such as monoclonal antibodies, radioimmunotherapy or antibody-drug conjugates [48,49].

Membrane/cytoplasmic CD276 immunoreactivity was frequently seen in CRC BM tumor and stromal cells. However, previously documented nuclear positivity was not noticed [50]. The latter was not reported by a recent study of 805 primary CRCs [51].

PRAME is a nuclear receptor and transcriptional regulator recognized by tumor-reactive cytotoxic T cells. PRAME expression highlights anti-PRAME immunotherapy targets [52]. Recent study reported PRAME positivity only in 1% of primary CRCs [53]. However, in CRC BMs, PRAME was expressed in almost 22% of cases. Thus, PRAME should be considered a potential therapeutic target.

An antibody-drug conjugate (H1D8-DC) targeted therapy is effective against CD44v5-positive intrahepatic cholangiocarcinoma cells and patient-derived xenograft models (ICC) [54]. Due to high expression of cathepsin B in ICC cells, the H1D8-drug conjugate is preferentially released in cancer cells but not in normal cells, thus inducing potent cytotoxicity at picomolar concentrations [54]. About one third of CRC BMs expressed CD44v5. Also, cathepsin is overexpressed in CRC [55]. Thus, CD44v5 could be a *bona fide* therapeutic target in CRC BMs.

In summary, this study showed that a considerable number of patients with CRC BMs could potentially benefit from individually tailored chemo-, molecularly targeted-, and immuno- therapy.

FIGURE LEGENDS

Figure 1. Immunohistochemistry of predictive biomarkers for cancer therapy. Diffuse and strong expression of: CK1 α 1 (A) in Case 50, CD44v5 (B) in Case 45, HER2 (C) in Case 15, SLFN11 (D), PRAME (E) and CD276 (F) in Case 5.

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Table 1. Demographic and clinicopathologic data of 60 CRC BMs.

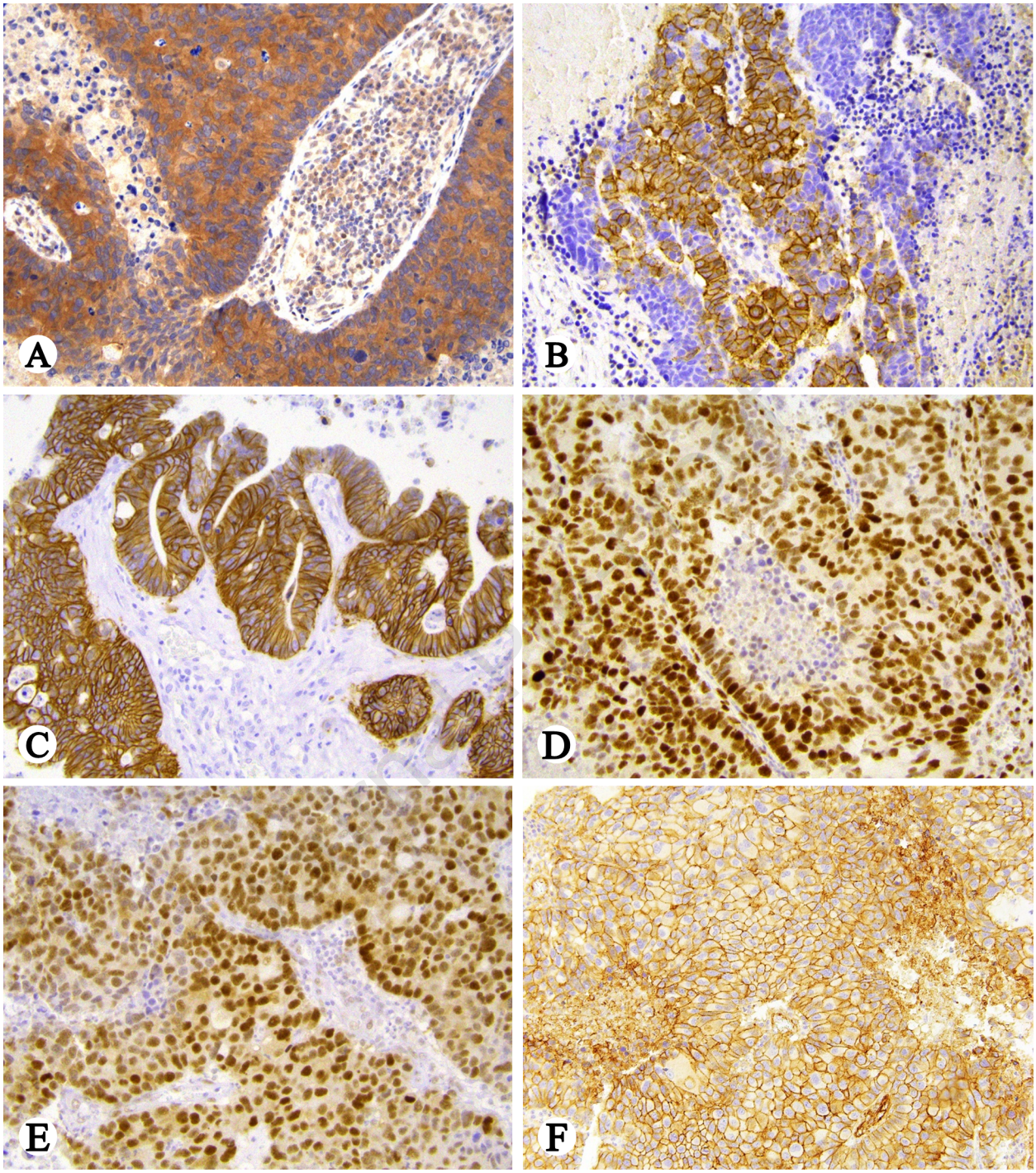
Clinical and pathological characteristics	n
Sex (median age)	
Female (67 y)	25 (41.7%)
Male (65 y)	35 (58.3%)
Primary tumor location	
Colon left side NOS	5 (8.3%)
Cecum	2 (3.3%)
Ascending	1 (1.7%)
Hepatic flexure	1 (1.7%)
Transvers	1 (1.7%)
Colon right side NOS	1 (1.7%)
Descending	1 (1.7%)
Sigmoid	6 (10%)
Rectosigmoid junction	1 (1.7%)
Rectum	20 (33.3%)
Unknown	21 (35%)
Site of brain metastasis	
Frontal lobe	11 (18.3%)
Occipital lob	4 (6.7%)
Parietal lobe	5 (8.3%)
Temporal lobe	6 (10%)
Frontoparietal region	1 (1.7%)
Occipitotemporal region	1 (1.7%)
Parietotemporal region	1 (1.7%)
Frontoparietotemporal region	1 (1.7%)
Cerebellum	12 (20%)
Cerebellar vermis	1 (1.7%)
Dura mater	2 (3.3%)
Unknown	15 (25%)
Histology	
Well-differentiated	1 (1.7%)
Moderately differentiated	24 (40%)
with mucinous component	1 (1.7%)
with signet ring cell component	1 (1.7%)
Mucinous	1 (1.7%)
Poorly differentiated	32 (53.3%)

Abbreviations: n-number of cases, y-years

Table 2. Summary of the results of immunohistochemical studies.

Antigens	n	Diffuse 3/2/1	Patchy 3/2/1	Focal 3/2/1	Scattered cells	Total
CDX2	60	54 (90%)	3 (5%)	2 (3.3%)	-	59 (8.3%)
CK20	60	26 (43.3%)	10 (16.7%)	10 (16.7%)	6 (10%)	52 (96.7%)
MLH1/PMS2 (loss)	60	3 (5%)	-	-	-	3 (5%)
MSH2 (loss)	60	-	-	-	-	0
MSH6 (loss)	54	-	-	-	-	0
β-catenin (loss of membrane staining)	59	4 (6.8%)	5 (8.5%)	-	-	9 (15.3%)
β-catenin (nuclear)	59	6 (10.2%)	-	14 (23.7%)	14 (23.7%)	34 (57.6%)
p53	57	42 (73.7%)	-	-	-	42 (73.7%)
p53 (loss)	57	10 (17.5%)	-	-	-	10 (17.5%)
CK1 α 1	60	10/25/22 (95%)	-	-	-	57 (95%)
CD44v5	60	3/0/0 (5%)	1/6/0 (11.7%)	1/0/10 (18.3%)	-	21 (35%)
HER2 (membrane)	60	2/1/0 (5%)	0/0/5 (8.3%)	-	-	8 (13.3%)
HER2 (nuclear)	60	1 (1.7%)	-	1 (1.7%)	-	2 (3.4%)
SLFN11	60	3 (5%)	2 (3.3%)	-	-	5 (5.3%)
MTAP (loss)	60	2 (3.3%)	2 (3.3%)	-	-	4 (6.7%)
PRAME	60	5/3/0 (13.3%)	5/0/0 (8.3%)	-	1(1.7%)	14 (23.3%)
CD279 (PD-1)	59	-	-	-	24[a]	-
CD274 (PD-L1)	59	-	-	-	12[a]	-
CD276 (tumor)	59	1/4/17 (37.3%)	-	0/3/1 (6.8%)	-	26 (44.1%)
CD276 (stroma)	55	20/13/17 (83.3%)	-	-	1[a]	50 (83.3%)

Abbreviations: n-number of cases, 3-strong-, 2-moderate-, 1-weak- staining, [a] -scattered immunocompetent cells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