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Research Article

Primary Mucinous Tumors of the Ovary: An Interobserver Reproducibility and Detailed Molecular Study Reveals Significant Overlap Between Diagnostic Categories

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ABSTRACT

Primary ovarian mucinous tumors represent a heterogeneous group of neoplasms, and their diagnosis may be challenging. We analyzed 124 primary ovarian mucinous tumors originally diagnosed as mucinous borderline tumors (MBTs) or mucinous carcinomas (MCs), with an emphasis on interobserver diagnostic agreement and the potential for diagnostic support by molecular profiling using a next-generation sequencing targeted panel of 727 DNA and 147 RNA genes. Fourteen experienced pathologists independently assigned a diagnosis from preset options, based on a review of a single digitized slide from each tumor. After excluding 1 outlier participant, there was a moderate agreement in diagnosing the 124 cases when divided into 3 categories ($\kappa = 0.524$, for mucinous cystadenoma vs MBT vs MC). A perfect agreement for the distinction between mucinous cystadenoma/MBT as a combined category and MC was found in only 36.3% of the cases. Differentiating between MBTs and MCs with expansile invasion was particularly problematic. After a reclassification of the tumors into near-consensus diagnostic categories on the basis of the initial participant results, a comparison of molecular findings between the MBT and MC groups did not show major and unequivocal differences between MBTs and MCs or between MCs with expansile vs infiltrative pattern of invasion. In contrast, HER2 overexpression or amplification was found only in

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5.3% of MBTs and in 35.3% of all MCs and in 45% of MCs with expansile invasion. Overall, HER2 alterations, including mutations, were found in 42.2% of MCs. *KRAS* mutations were found in 65.5% and *PIK3CA* mutations in 6% of MCs. In summary, although the diagnostic criteria are well-described, diagnostic agreement among our large group of experienced gynecologic pathologists was only moderate. Diagnostic categories showed a molecular overlap. Nonetheless, molecular profiling may prove to be therapeutically beneficial in advanced-stage, recurrent, or metastatic MCs.

Introduction

Primary mucinous tumors of the ovary represent a heterogeneous group of neoplasms composed of gastrointestinal-type cells and are grouped into benign, borderline, and malignant categories.[1–5] Approximately 80% of mucinous tumors are classifiable as benign mucinous cystadenomas/adenofibromas (MAs), followed in decreasing frequency by mucinous borderline tumors (MBTs) and mucinous carcinomas (MCs). The diagnosis of MA is usually straightforward; a potentially problematic area is MA with focal epithelial proliferation, which is arbitrarily defined as an MA with <10% of epithelial areas exhibiting proliferation with features of an MBT. The diagnosis of an MBT with "typical" features is generally not problematic either; however, some cases can show high-grade nuclear atypia, markedly increased epithelial proliferation, a combination of these 2 features, or other features that place them within the "gray zone" between MBTs and MCs with expansile invasion.[2,6–8] Cases with high-grade nuclear atypia but otherwise typical MBT architecture are classified as MBTs with intraepithelial carcinoma.[2] Primary ovarian MC is defined by the presence of invasion with at least 5.0 mm in the largest linear extent. An invasion of <5.0 mm in a maximum dimension is defined as microinvasion, when the cells display only mild-to-moderate atypia and resemble those of the surrounding MBT, or as microinvasive carcinoma when the cells display architectural and cytologic features of invasive carcinoma.[9–11] Two types of invasion are recognized in MCs: expansile or confluent (nondestructive) and infiltrative (destructive).[1,4,12] The expansile pattern is the more common type and is characterized by marked crowding of well-formed glands or interconnecting epithelial branching and papillary proliferation, with a substantial reduction or absence of intervening stroma and forming a maze-like pattern; a desmoplastic stromal reaction is usually absent. In contrast, the infiltrative type of invasion is characterized by the destructive growth of irregular nests, glands, or isolated tumor cells with malignant cytologic features and is usually associated with a desmoplastic stromal reaction. An MC with infiltrative invasion has been associated with a worse prognosis than an MC with expansile invasion.[12–14] Given the overlap in morphologic features, the possibility that an MC in the ovary is actually metastatic to this site is usually a diagnostic consideration whenever a tumor displays an infiltrative type of invasion. The nodular growth pattern, which may be conceptualized as another pattern of invasion, is typically associated with ovarian metastases of extraovarian MCs.[1,4]

Preliminary evidence suggests that there are diagnostic issues related to the classification of subsets of ovarian mucinous neoplasms. The reproducibility of the distinction between MBTs with or without intraepithelial carcinoma and MCs with expansile invasion seems to be suboptimal. Furthermore, there are problems in the distinction between an MC with expansile invasion and an MC with infiltrative invasion because, in both scenarios, the lesions may coexist.[2,8,13]

The primary goal of our study was to assess diagnostic reproducibility among a group of 14 experienced pathologists (P.D., T.B., J.D., P.F., O.F., J.H., J.L., S.F.L., R.M., K.N., N.S., S.S., M.Š., W.G.M.) in their classification of 124 primary ovarian mucinous tumors. In addition, the controversial issue of MC grading was discussed on the basis of a survey of the approaches taken by the participants in their routine practices. Second, we compared the molecular findings between MBTs and MCs with the aim of finding adjunct markers for a differential diagnosis, the prediction of outcome, and targeted therapy.

Material and Methods

Case Selection

The cases of MCs were retrieved from the archives of the following institutions: Department of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague; Department of Pathology, Charles University, Third Faculty of Medicine, University Hospital Královské Vinohrady; Department of Oncological Pathology, Masaryk Memorial Cancer Institute; Department of Pathology, University Hospital Brno and Medical Faculty, Masaryk University; The Fingerland Department of Pathology, Charles University, Faculty of Medicine Hradec Králové and University Hospital Hradec Králové; Department of Pathology, Faculty of Medicine, University of Debrecen; and Šikl's Department of Pathology, The Faculty of Medicine and Faculty Hospital in Pilsen, Charles University, Pilsen, Czech Republic. Cases of MBTs were retrieved from the archives of the Department of Pathology, First Medical Faculty and General University Hospital in Prague. For all cases with available blocks and slides, the authors carefully reviewed all clinicopathologic data and examined their immunohistochemical (IHC) profiles (see the section "Immunohistochemistry") to exclude other histologic types of primary ovarian neoplasms and to rule out the possibility of metastasis. All MCs with insufficient clinical data or workup necessary to exclude an ovarian metastasis were excluded from the study. Ultimately, 124 cases were selected for further analysis.

From each tumor, 1 representative section was selected and scanned as whole-slide image using the Panoramic MIDI (3DHISTECH) scanner. The slide selection was performed by a single experienced gynecologic pathologist (P.D.), and in heterogeneous cases, the "worst area" slide (most morphologically "worrying" area) was selected. The scanned slides were uploaded to a Virtual Case Center, from which they were available to all 14 participants of the study. The participants were asked to assign the tumors to the following diagnostic categories: "benign MA," "benign MA with focal epithelial proliferation," "MBT," "MBT with intraepithelial carcinoma," "MBT with microinvasion," "MBT with microinvasive carcinoma," "MC with expansile invasion," "MC with expansile invasion and infiltrative microinvasion," "MC with expansile and

infiltrative invasion," "MC with infiltrative invasion," "MC with indeterminate type of invasion," "equivocal between MA and MBT," and "equivocal between MBT and MC." Moreover, each observer filled out a questionnaire with 4 questions concerning the issue of grading of MCs (Supplementary Table S1). For the molecular analysis, agreement among the participants was taken into account. Only tumors in which at least 9 observers agreed on the 2 main diagnostic categories (MA+MBT and MC) were included in the molecular part of the study. Further subclassification of MCs was based on the majority opinion of the participants. The distribution of cases in each category is detailed in Figure 1.

Immunohistochemical Analysis

Immunohistochemistry was performed on tissue microarrays (TMAs) using 4-μm–thick sections of formalin-fixed and paraffin-embedded (FFPE) tissues. For the construction of TMAs, eligible areas of each tumor were identified, and 2 tissue cores (each 2.0 mm in diameter) were taken from the donor block using the TMA instrument TMA Master (3DHISTECH Ltd). If the cores from a case did not contain representative tumor areas or were lost during processing, new cores were taken for new TMAs. For 1 MC with infiltrative invasion, whole-tissue sections were used because the TMA approach was not technically optimal because of a small tumor size. For the confirmation of mucinous differentiation and for exclusion of metastases, the following antibodies were analyzed in each tumor, in accordance with published evidence and as outlined in the fifth edition of the World Health Organization classification: cytokeratin 7, cytokeratin 20, estrogen receptor, progesterone receptor, PAX8, CDX2, and SATB2.[10,15] Additionally, HER2 expression was assessed in each case. The clones, manufacturers, dilutions, and staining instruments for all antibodies are summarized in Supplementary Table S2.

Immunohistochemical Scoring

All cases were scored by at least 2 experienced pathologists (P.D., M.B., or K.N.). Cases were classified on the basis of the overall percentage of positive cells as negative (entirely negative or <5% of positive tumor cells) or positive (5%-100% of positive tumor cells). *HER2* scoring was performed according to the 2018 ASCO/CAP guidelines for breast carcinoma.[16] HER2 overexpression was defined as a score 3+ immunoreacticvity in >10% of tumor cells.

Isolation of DNA and RNA for Next-Generation Sequencing

Genomic DNA and total RNA were isolated from FFPE tissues of the tumors using the Quick-DNA/RNA FFPE Miniprep

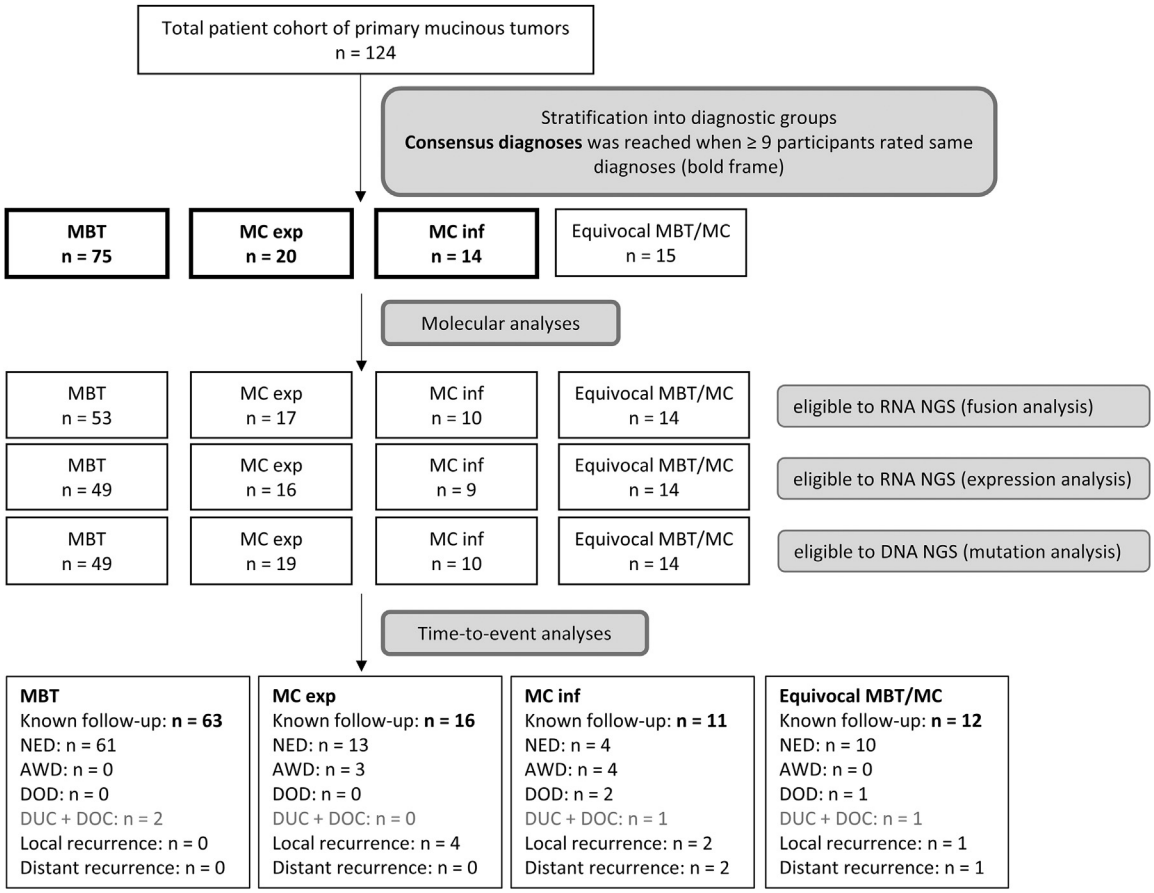

Figure 1.
CONSORT (Consolidated Standards of Reporting Trials) diagram. AWD, alive with disease; DOC, death of other cause; DOD, death of disease; DUC, death of unknown cause; equivocal MBT/MC, cases without consensus diagnoses classifies as equivocal between MBT and MC; MBT, mucinous borderline tumor; MC exp, mucinous carcinomas with expansile invasion; MC inf, mucinous carcinomas with infiltrative invasion; NED, no evidence of disease.

Kit (Zymo Research) according to the manufacturer's protocol. Isolated DNA was stored at −20 °C and total RNA at −80 °C until the preparation of next-generation sequencing (NGS) libraries.

DNA Next-Generation Sequencing Analysis

The sequence-capture NGS analysis of DNA was performed for all qualitatively sufficient cases (92/124; 74.2%) using the KAPA HyperPlus kit (according to KAPA HyperCap Workflow v3.0; Roche) and a panel of hybridization probes against multiple targets of cancer relevant genes (727 genes or gene parts; 2097 kbp of target sequence including 1708 kbp of coding regions; Roche; Supplementary File S1). The prepared sample libraries were pair-end sequenced by the NextSeq 500 instrument (Illumina) using the NextSeq 500/550 High Output Kit v2.5 (Illumina). Biostatistical evaluation using the NextGENe software (Softgenetics) and interpretation of DNA variants were performed as previously described.[17]

Briefly, all frameshift, no-start and no-stop splice variants in the consensus splice sites, and nonsense variants and missense variants, known as pathogenic and/or likely pathogenic (class 4/5 mutations), respectively, according to ClinVar database were considered deleterious. Detailed pipelines of all NGS data analysis together with module settings are available on request. The analysis does not allow the distinction between somatic and germline variants. OncoKB (https://www.oncokb.org/) and My Cancer Genome databases (https://www.mycancergenome.org/) were searched for the clinical significance of the detected mutations in selected genes with respect to therapeutic actionability.[18] *TP53* variants were classified according to https://p53.iarc.fr/, ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and https://www.cancerhotspots.org/. The size of the sequenced panel enabled us to assess the tumor mutation burden (TMB; the number of mutations per 1 mega base; mut/Mb); however, the TMB was calculated only for samples with ≥40% tumor cells. All synonymous and nonsynonymous variants with an allele frequency of ≥10% were counted. Furthermore, potential germline variants according to databases of known germline polymorphisms (including Single Nucleotide Polymorphism database and Exome Aggregation Consortium) and known or probable driver mutations (according COSMIC and ClinVar database) were determined. The resulting mutation number was recalculated to 1 Mb.

RNA Next-Generation Sequencing Analysis

Total RNA samples were processed according to the KAPA RNA HyperPrep Kit protocol (Roche; input 300 ng where available; denaturation/fragmentation 85 °C for 2 minutes; 11 cycles of PCR). In the samples of sufficient quality (94/124; 76%), the target sequences were enriched by the standard KAPA HyperCap Workflow v3.0 (Roche) using a custom panel focused on the pan-cancer markers and potential fusion genes (147 genes; 373 kbp of the target DNA sequence; Roche) (Supplementary File S1). The final libraries were pair-end sequenced by the NextSeq 500 instrument using 300 cycle chemistry kits (Illumina), with a target of 10 million single reads.

The sequencing data were analyzed using the CLC Genomics Workbench v21.0.5. (CLC GW; Qiagen) by an in-house pipeline, including the targeted RNA-Seq expression analysis (RNA-Seq Analysis module) and detection of fusion genes (Detect and Refine Fusion Genes module). The bioinformatics pipeline and module settings are available upon request.

All fusions identified by the CLC GW were manually checked, filtered, and confirmed using IGV v2.11.3 (Broad Institute). Only the fusions meeting the following criteria were considered true fusions: (1) fusions involving protein-coding genes with standard exon-exon junctions, with substantial expression compared with those of other samples in the respective regions; (2) ≥10% of reads supporting fusion presence (crossing reads) out of read counts at respective location. Frequently repeated fusions, fusions of genes from the same gene family, or transcriptional read through were excluded and considered as artifacts.

Expression profiling was performed using Heat Map for RNA-Seq module in CLC GW with Manhattan distance and Complete linkage settings. Eleven genes (namely *ALK*, *CYP19A1*, *H3F3C*, *NRG1*, *NTRK1/3*, *NUTM1*, *PAX3*, *PRKCB*, *RET*, and *ROS1*) with low expression in all samples (under the 100 TPM relative to the expression of the used panel) were discarded to reduce the background noise. The nomenclature of the detected mutations followed the Human Genome Variation Society recommendations (https://varnomen.hgvs.org/).

Fluorescence In Situ Hybridization Analysis for HER2

All cases with HER2 2+ were analyzed for amplification by fluorescence in situ hybridization using 4-μm−thick whole-tissue sections of FFPE tumor tissue and ZytoLight SPEC ERBB2/CEN 17 Dual Color Probe (Z-2077; ZytoVision GmbH) according to the manufacturer's protocol. A HER2/CEP17 ratio ≥ 2.0 was considered as amplification.

Statistical Analyses

All statistical tests were performed using the program R (version 4.0.2; https://www.r-project.org/) or Statistica (TIBCO). Correlation between immunohistochemistry and clinicopathologic characteristics was performed using the Pearson χ^2 test, Fisher exact test, or nonparametric analysis of variance approach (H score as a continuous variable). Differences of the expression of markers between the 2 diagnostic groups were evaluated using the Mann-Whitney *U* test.

Interobserver agreement was determined using percentage agreement and κ statistic. The rating classification included 3 main diagnostic categories: MA, MBT, and MC. For the assessment of diagnostic agreement, cases classified as MAs with focal epithelial proliferation were included in the category of MBTs because the study allowed only the assessment of a single slide; therefore, it was not possible to determine the true extent of epithelial proliferation. The level of agreement among the participants was evaluated using Fleiss κ coefficients. These analyses were conducted using the "irr" library implemented in the R software (https://www.r-project.org/). Consistent with the findings of a previous literature on the level of agreement, κ coefficients were interpreted as poor (<0.00), slight (0.01-0.20), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80), and almost perfect (0.81-1.00).[19]

The stratification of the cases into the 2 main diagnostic categories (MA and MBT vs MC) was based arbitrarily on the agreement between at least 9 of the 14 participants, which was achieved in 109 cases. The diagnostic agreement was primarily evaluated among all 14 participants. Because the results by 1 participant differed in a substantial number of cases from the

other participants (48% discordant diagnoses from the consensus compared with 1%-18% [median, 8.1%] with other participants), we considered this participant as an "outlier" and calculated the data after excluding this participant from the analysis of interobserver agreement. Based on the stratification of the tumors into the diagnostic categories, we performed time-to-event analyses with 3 outcomes: relapse-free survival (RFS, the period from the date of diagnosis to the date of relapse of disease or death), local recurrence–free survival (LFS, the period from the primary diagnosis until the first local recurrence), and distant metastasis–free survival (MFS, the period from the primary diagnosis until the diagnosis of the first distant metastasis). Because of an insufficient number of events, the overall survival (the period from the date of diagnosis to the date of recorded death) was not calculated. Survival analyses were plotted using the Kaplan-Meier model, and the differences between curves were tested for significance using the log-rank test.

Results

Clinicopathologic data are summarized in Supplementary Table S3.

Interobserver Reproducibility

The results of the interobserver reproducibility study are detailed in Table 1. Among all 14 participants, there was a moderate agreement in diagnosing the 124 cases when divided into 3 categories ($\kappa = 0.436$, for MA vs MBT vs MC). After excluding the outlier, the agreement among the 13 remaining pathologists improved, although it was still moderate ($\kappa = 0.524$).

Interobserver agreement for the subgroup of MA and MBT cases reached only very poor agreement both for 5 categories (all 14 pathologists: $\kappa = 0.096$; 13 pathologists after the exclusion of the outlier: $\kappa = 0.122$) and for the comparison between MBTs with intraepithelial carcinoma and all remaining MBT cases (all 14 pathologists: $\kappa = 0.075$; 13 pathologists after the exclusion of the outlier: $\kappa = 0.109$).

Perfect agreement (100% match) for the distinction of MA/MBT (as a combined category) from MC was found in only 27 of 124 (21.3%) cases, including 19 MBT and 8 MC (3 with expansile and 5 with infiltrative invasion) cases, for all 14 pathologists and in 45 of 124 (36.3%) of the cases, including 34 MBT and 11 MC (4 with expansile and 7 with infiltrative invasion) cases, after excluding the outlier for 13 pathologists. Representative images of the tumors are shown in Figures 2-4 and Supplementary Figure S1.

After applying our criteria for diagnostic agreement, the study cohort consisted of 75 MBT cases, 34 MC cases (20 with expansile and 14 with infiltrative invasion), and 15 tumors classified as equivocal between MBTs and MCs.

Immunohistochemical Findings

The expression of the IHC markers for confirmation of the mucinous differentiation and exclusion of metastases was similar between MBTs and MCs. No cases were excluded from the study after the IHC analysis. The results are summarized in Supplementary Table S4.

HER2 immunoreactivity was scored as 0 in 71 tumors, 1+ in 24 tumors, 2+ in 16 tumors, and 3+ in 13 tumors. A statistically significant difference regarding the HER2 status was found between MBTs and MCs. HER2 overexpression (3+) or amplification (detected by fluorescence in situ hybridization) was found in 4 of 75 (5.3%) MBT cases and in 12 of 34 (35.3%) MC cases ($\chi^2 = 16.8$, $df = 1$, $P < .001$). Moreover, a difference was present between the 2

Table 1
Interobserver agreement of the diagnoses

Groups	Diagnosis	No. of cases	κ	SE	95% CI	z	P value	Result
Among all pathologists (14 observers)	All diagnoses: 2 categories[a]	124	0.445	0.008	0.416-0.448	52.1	<.001	Moderate agreement
	All diagnoses: 3 categories[b]	124	0.436	0.009	0.418-.0428	52.3	<.001	Moderate agreement
	MBT: 5 categories[c]	75	0.096	0.007	0.077-0.104	14.3	<.001	Slight agreement
	MBT: 2 categories[d]	75	0.075	0.006	0.072-0.101	8.1	<.001	Slight agreement
	MC: 5 categories[e]	34	0.287	0.012	0.321-0.368	30.4	<.001	Fair agreement
	MC: 3 categories[f]	34	0.330	0.017	0.358-0.426	26.2	<.001	Fair agreement
Among all pathologists, except for 1 (13 observers)[g]	All diagnoses: 2 categories[a]	124	0.534	0.016	0.420-.0435	58.2	<.001	Moderate agreement
	All diagnoses: 3 categories[b]	124	0.524	0.017	0.418-0.442	57.8	<.001	Moderate agreement
	MBT: 5 categories[c]	75	0.122	0.007	0.104-0.133	16.8	<.001	Slight agreement
	MBT: 2 categories[d]	75	0.109	0.007	0.102-0.125	10.8	<.001	Slight agreement
	MC: 5 categories[e]	34	0.330	0.013	0.368-0.420	32.4	<.001	Fair agreement
	MC: 3 categories[f]	34	0.387	0.019	0.417-0.493	27.9	<.001	Fair agreement

MBT, mucinous borderline tumor; MC, mucinous carcinoma.

[a] All diagnoses from the complete data set included 2 categories: (1) benign mucinous cystadenoma/adenofibroma + MBT, (2) MC.

[b] All diagnoses from the complete data set included 3 categories: (1) benign mucinous cystadenoma/adenofibroma, (2) MBT, and (3) MC.

[c] MBT included 5 categories: (1) benign mucinous cystadenoma/adenofibroma with focal epithelial proliferation, (2) MBT, (3) MBT with microinvasive carcinoma, (4) MBT with microinvasion, and (5) MBT with intraepithelial carcinoma.

[d] MBT included 2 categories: (1) benign mucinous cystadenoma/adenofibroma with focal epithelial proliferation + MBT + MBT with microinvasive carcinoma + MBT with microinvasion and (2) MBT with intraepithelial carcinoma.

[e] MC included 5 categories: (1) MC with expansile invasion, (2) MC with expansile invasion and infiltrative microinvasion, (3) MC with expansile and infiltrative invasion, (4) MC with infiltrative invasion, and (5) MC with indeterminate type of invasion.

[f] MC included 3 categories: (1) MC with expansile invasion + MC with expansile invasion and infiltrative microinvasion, (2) MC with expansile and infiltrative invasion + MC with infiltrative invasion, and (3) MC with indeterminate type of invasion.

[g] One outlier (the rater who markedly differed in the diagnosis from all other pathologists) was excluded from the analysis.

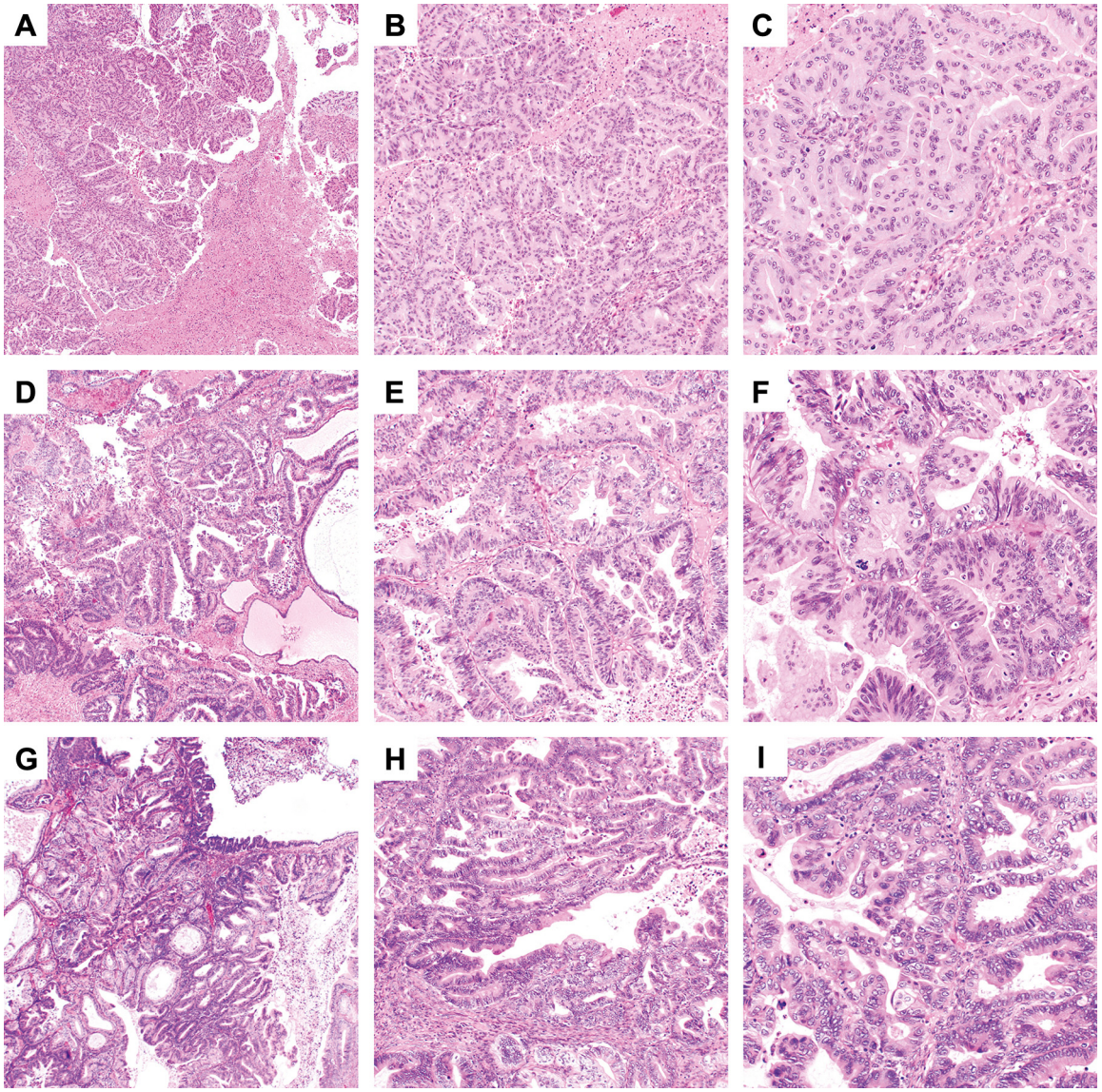

Figure 2.
Ovarian mucinous carcinoma with expansile invasion: cases with 100% agreement among all observers. Case 10 (A-C, ×40, ×100, and ×200, respectively); case 44 (D-F, ×40, ×100, and ×200, respectively), and case 83 (G-I, ×40, ×100, and ×200, respectively).

MC categories but was not statistically significant because of the small number of cases. In MCs with infiltrative invasion, HER2 overexpression or amplification was present in 21.4% (3/14) compared with 45% (9/20) in MCs with expansile invasion ($\chi^2 = 2.0$, df = 1, $P = .157$).

Molecular Findings

The DNA NGS analysis of 92 eligible cases revealed pathogenic or likely pathogenic (class 4/5) mutations in 76 of 727 analyzed genes (Fig. 5). The frequencies of mutations in the 10 most frequently affected genes are summarized in Table 2, together with literary data. The most frequently affected genes by class 4/5 mutations were *KRAS* (65.3% of MBT [32/49], 73.7% of MC [14/19], and 57.1% of cases equivocal between MBT and MC [8/14]) and *TP53* (42.9% of MBT [21/49], 69% of MC [20/29], and 64.3% of cases equivocal between MBT and MC [9/14]). Seven of 50 (14%) cases with *TP53* mutations were in the nonhotspot regions (6 in MBT and 1 in MC). A forthcoming study will focus in detail on the correlation between p53 IHC and *TP53* mutation status. Of the 92 tumors, 29 (32%) harbored both *KRAS* and *TP53* mutations. Furthermore, 11 of 15 cases with mutations in *CDKN2A* harbored a *TP53* mutation, and 5 of these cases had concurrent *KRAS* mutations. *KRAS* mutations frequently occurred together with any of the recurrently mutated genes including *ARID1A*, *ATM*, *BRAF*, *CDKN2A*, *ELF3*, *HER2*, *PIK3CA*, *RNF43*, and *TP53*. Overall, 76 of 92 (83%) cases revealed altered RAS/RAF/MAPK or PI3K/Akt/mTOR signaling pathways. TMB was evaluable in 43 cases. The average TMB was low and similar for both MBT and MC subgroups, with 1.8 mut/Mb (range, 0-4) in 49 MBT cases and 2.3 mut/Mb (range, 0-5) in 29 MC cases. Copy number variation (CNV) analyses were not performed because of low DNA quality not suitable for reliable CNV testing.

The RNA NGS analysis of the selected genes was performed for 94 cases. The expression profiles were compiled for 88 cases,

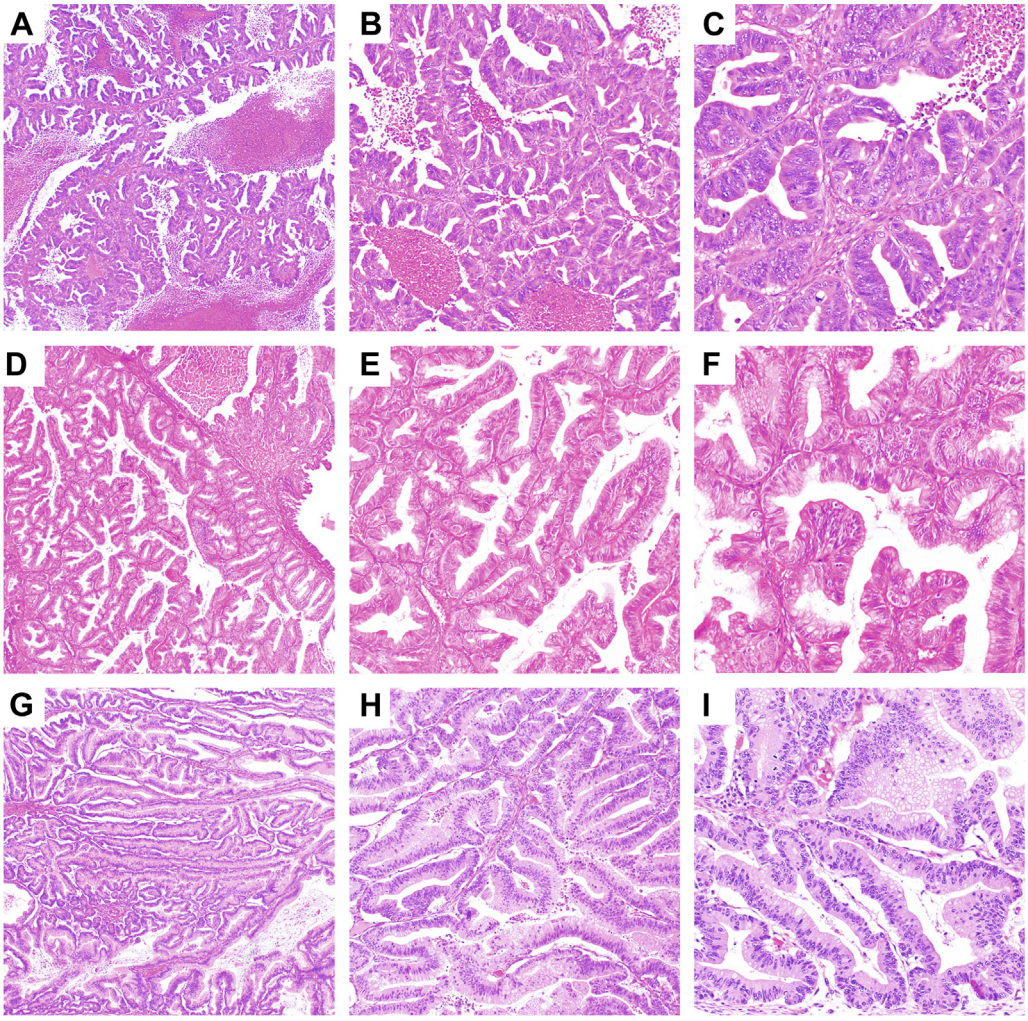

Figure 3.
Ovarian mucinous carcinoma with expansile invasion: cases with agreement among most observers. Case 52 (A-C, ×40, ×100, and ×200, respectively), diagnostic agreement of 12 experts; case 84 (D-F, ×40, ×100, and ×200, respectively), diagnostic agreement of 10 experts; and case 92 (G-I, ×40, ×100, and ×200, respectively), diagnostic agreement of 12 experts.

which reached sufficient quality (≥5 million single reads), including 9 MCs with infiltrative growth, 16 MCs with expansile growth, 49 MBTs, and 14 equivocal between MBT and MC cases. The results of the molecular analysis did not show any significant stratification of the samples (Fig. 6). Gene fusion analysis was performed in 94 cases (53 MBT, 17 MC with expansile growth, 10 MC with infiltrative growth, and 14 equivocal cases). The gene fusion TFG::ADGRG7 (NM_001007565.2:r.1_453::(NM_032787.3):r.372_3128 was found in 6 cases, including 2 of 17 (12%) MCs with expansile invasion and 4 of 53 (8%) MBTs. Two novel fusion candidates BAP1::CACNA1D and MAP2K4::PIK3C2G were identified in 2 MBT cases: (NM_004656.4}:r.1_789::(NM_000720.4):r.4292_9429 and (NM_003010.4}:r.1_228::(NM_004570.5):r.1874_4844.

Outcome Analysis

As shown in Figure 7, there was statistically significant worse RFS for patients with MCs with infiltrative invasion compared with their counterparts with MCs with expansile invasion, equivocal cases between MBT and MC, and MBTs ($P < .001$). When comparing RFS between the 2 MC categories, we still observed a statistically significant worse survival for MC with infiltrative invasion cohort, compared with that of the MC with expansile invasion cohort ($P = 0.046$). In contrast, LFS and MFS did not show any significant differences between the MC with expansile invasion and MC with infiltrative invasion cohorts (LFS, $P = .584$; MFS, $P = .130$).

Discussion

The categorization of primary ovarian mucinous tumors is not always straightforward. After confirming the mucinous differentiation of an ovarian tumor and excluding a metastasis, other diagnostic challenges may still remain. With the exception of benign tumors, the overall interobserver variability in the diagnosis of ovarian mucinous tumors seems to be high, but the data from the literature are limited.[13,20] This is the largest study to date investigating the interobserver variability of mucinous ovarian tumors by expert pathologists. Although the diagnostic criteria are well-described, the agreement among our large group of

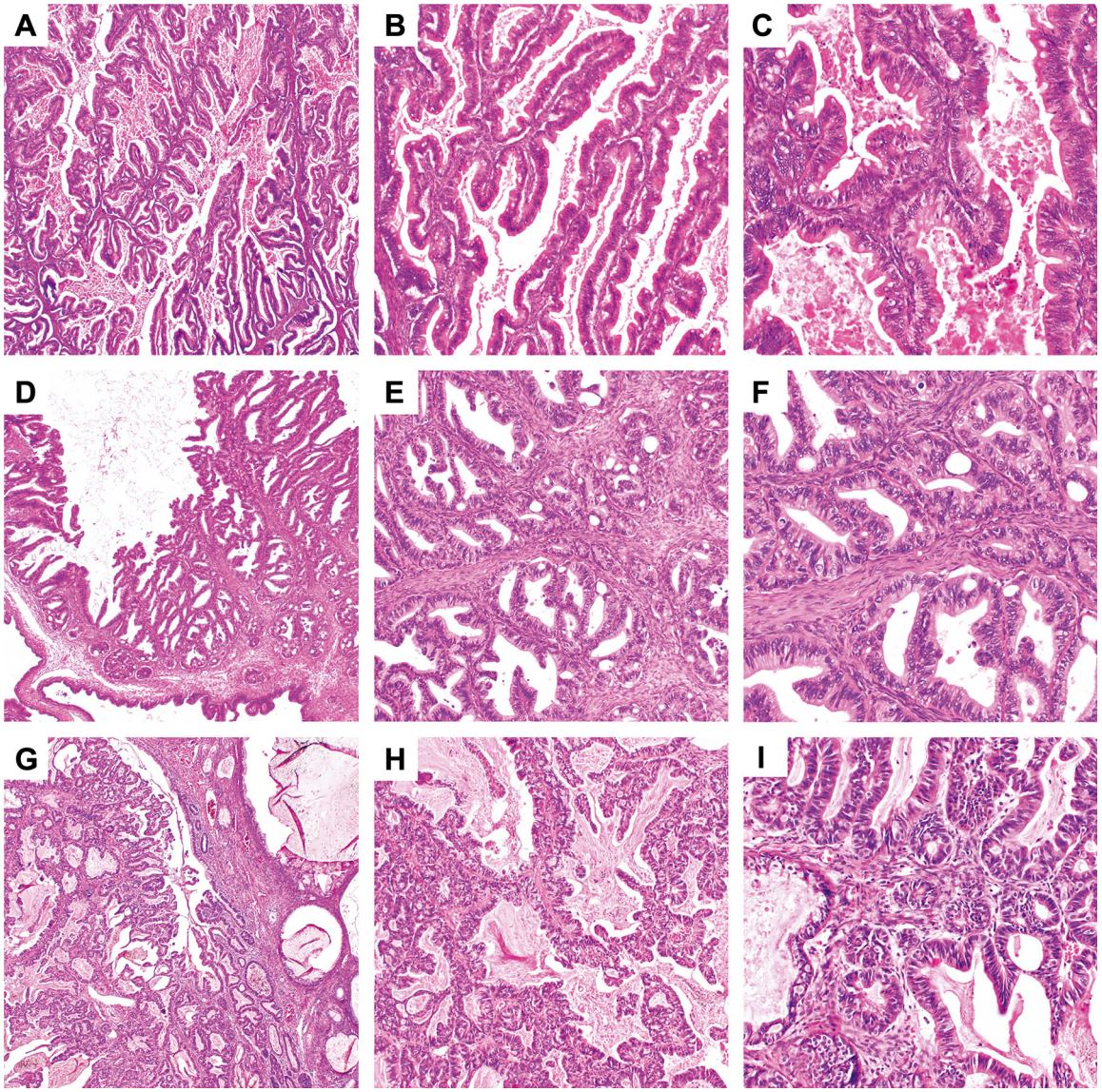

Figure 4.
Ovarian mucinous neoplasms without diagnostic agreement. Case 55 (A-C, ×40, ×100, and ×200, respectively), 6 experts classified the tumor as mucinous borderline tumor (MBT), 5 as mucinous carcinoma (MC), and 3 as equivocal. Case 29 (D-F, ×40, ×100, and ×200, respectively), 5 experts classified the tumor as MC, 8 as MBT, and 1 as equivocal. Case 6 (G-I, ×40, ×100, and ×200, respectively), 8 experts classified the tumor as MBT and 5 as MC.

experienced gynecologic pathologists was only moderate (κ = 0.436). Differentiating between MBTs and MCs was the most problematic issue. Most consensus diagnosis did not show any molecular stratification, with the exception of HER2 aberration. However, clinical outcomes showed a poorer survival for MCs than for MBTs and tumors classified as equivocal between MC and MBT.

One particularly problematic issue is the differential diagnosis between MBTs and MCs with expansile invasion, and there is limited evidence in the literature concerning the interobserver variability. Generally, the interobserver agreement seems to be better when the number of investigators is small. In a study of 73 ovarian mucinous tumors with 6 participants, the agreement was moderate (κ = 0.56), whereas in another study of 79 tumors, a concordance of 85% (κ = 0.78) between 2 participants was found.[13,20] However, as the authors noted, 18% of the cases that had been initially diagnosed as MCs were reclassified by both participants as MBTs.[13] In our study, it was not possible to assess with certainty the concordance rate for distinguishing between MCs with expansile invasion and MBTs. For such an analysis, we would have to exclude cases that some observers rated as MCs with infiltrative invasion; therefore, the data set would be artificially reduced to 56 cases. Nonetheless, our results highlight the fact that the diagnostic criteria for the distinction between MBTs and MCs with expansile invasion are problematic and that their application by pathologists is associated with poor reproducibility. The current approach to the classification of ovarian mucinous tumors seems to be problematic given that most tumors classified as MBTs are biologically benign and those MBTs in the gray zone between MBTs and MCs that recur or even metastasize are difficult to be recognized and to be classified. However, histology remains the cornerstone in the diagnosis of primary mucinous ovarian tumors. Although the morphologic criteria, especially for distinction between MBTs and MCs with expansile invasion, are poorly reproducible because of the overlap not only in

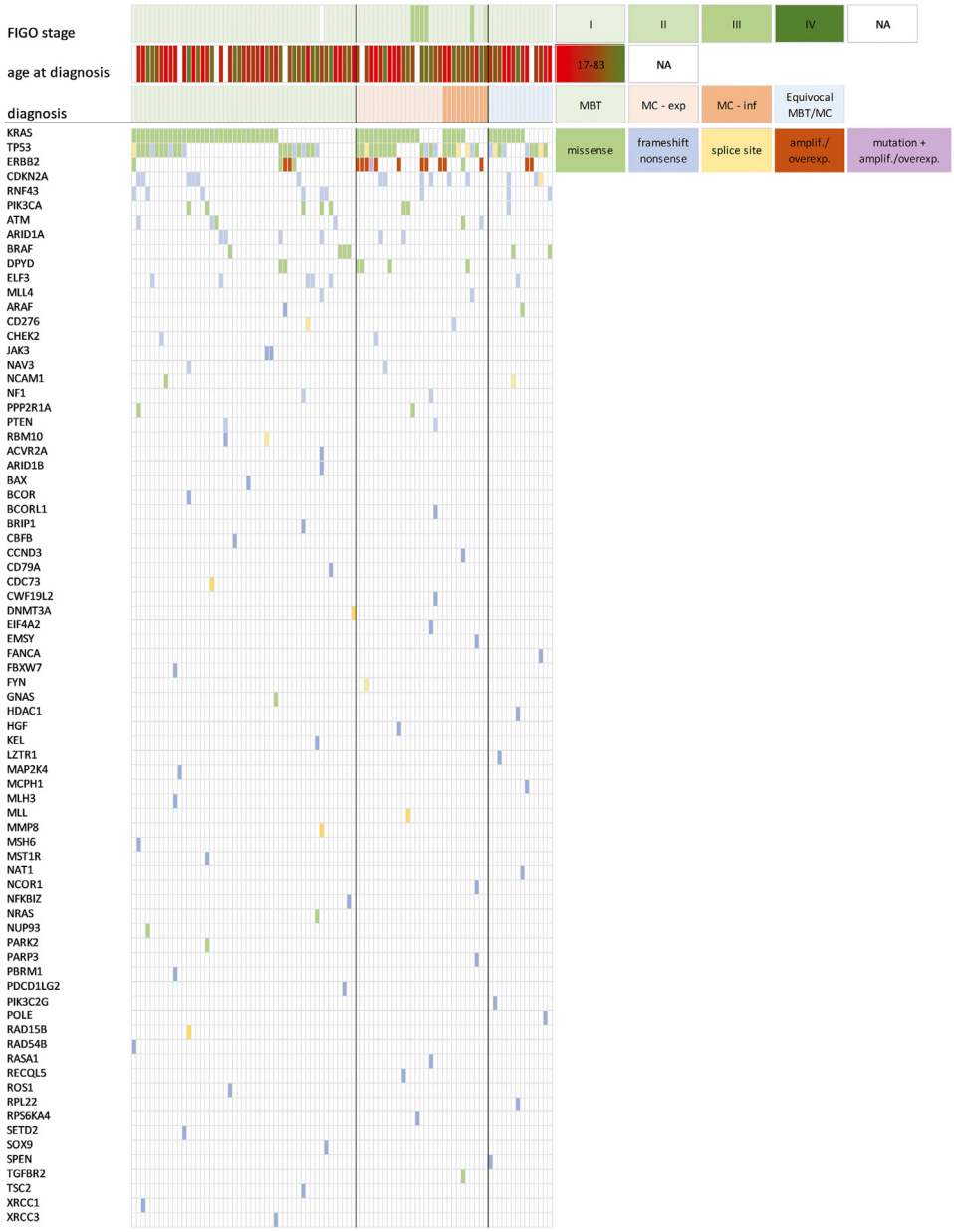

Figure 5.
Spectrum of detected pathogenic or likely pathogenic mutations in 92 mucinous tumors in the context of clinicopathologic characteristics. The biostatistical next-generation sequencing data analysis was performed using the NextGENe software (Sofgenetics). Only pathogenic or likely pathogenic mutations (classes 4 and 5, respectively) were reported. FIGO, International Federation of Gynecology and Obstetrics; equivocal MBT/MC, cases without consensus diagnoses classifies as equivocal between MBT and MC; MBT, mucinous borderline tumor; MC exp, mucinous carcinomas with expansile invasion; MC inf, mucinous carcinomas with infiltrative invasion; NA, not available.

morphology but also in molecular and IHC features, attempts at more precise definition of these criteria allowing more reproducible classification would probably fail.

Several studies suggest that the biological behavior of MCs with expansile invasion is more favorable than that of MCs with infiltrative invasion.[12,21–23] In addition, the growth pattern has been suggested as a possible grading system for MC.[14] In contrast, other studies have suggested that some patients with MCs and pure expansile growth can have poor clinical outcomes.[7,21,24] In 1 study, the authors summarized their experience with 4 cases of stage I MC with expansile invasion and reviewed the available literature. They found that approximately 5% of stage I MCs lacking infiltrative invasion behaved in a malignant fashion.[24] Another study showed recurrence with poor outcome in 23.1% of MCs with pure expansile invasion.[25] In our study, the prognosis was best for tumors classified as MBTs, which in all cases behaved in a benign fashion, followed by cases equivocal between MBTs and MCs, MCs with expansile invasion, and MCs with infiltrative invasion. Two of 12 (17%) equivocal cases between MBTs and MCs recurred, and one of these patients died of the disease. Three of 16 (18.7%) patients with MCs with pure expansile invasion experienced local recurrence, and none of these patients died of the disease. The prognosis of MCs with infiltrative invasion was the worst, with 4 of 11 (36%) patients developing recurrent disease and 2 of 11 (18%) patients dying of disease.

Table 2
The frequency of mutations in the 10 most frequently mutated genes in 92 primary mucinous tumors and comparison with that reported in the literature

Gene	Mucinous all, n/N (%)	MBT, n/N (%)	MC all, n/N (%)	MC exp, n/N (%)	MC inf, n/N (%)	Equivocal MBT/MC, n/N (%)	MBT literature, n/N (%), range	MC literature, n/N (%), range
KRAS	59/92 (64.1)	32/49 (65.3)	19/29 (65.5)	14/19 (73.7)	5/10 (50)	8/14 (57.1)	83/119 (69.7), 20-92	237/365 (63.2), 44-76
TP53	50/92 (54.3)	21/49 (42.9)	20/29 (69)	13/19 (68.4)	7/10 (70)	9/14 (64.3)	12/123 (9.8), 0-18	177/319 (55.5), 0-64
CDKN2A	15/92 (16.3)	6/49 (12.2)	6/29 (20.7)	4/19 (21.1)	2/10 (20)	3/14 (21.4)	30/65 (46.2),[a] 19-64	158/232 (68.1),[a] 19-77
RNF43	9/92 (9.8)	6/49 (12.2)	1/29 (3.4)	1/19 (5.3)	0/10 (0)	2/14 (14.3)	5/49 (10.2), 9-11	29/225 (12.9), 12-21
PIK3CA[b]	8/92 (8.7)	5/49 (10.2)	2/29 (6.9)	2/19 (10.5)	0/10 (0)	1/14 (7.1)	8/63 (12.7), 0-15	22/260 (8.5), 0-14
ATM	6/92 (6.5)	4/49 (8.2)	2/29 (6.9)	0/19 (0)	2/10 (20)	0/14 (0)	2/27 (7.4)	12/181 (6.6)
ARID1A[b]	6/92 (6.5)	4/49 (6.1)	2/29 (6.9)	2/19 (10.5)	0/10 (0)	0/14 (0)	3/27 (11.1)	19/199 (9.5), 0-10
HER2 (mut)	5/92 (5.4)	3/49 (4.1)	2/29 (6.9)	1/19 (5.3)	1/10 (10)	0/14 (0)	1/134 (0.7)	0/195 (0)
HER2 (o/a)	18/124 (14.5)	4/75 (5.3)	12/34 (35.3)	9/20 (45)	3/14 (21.4)	2/15 (13.3)	21/326 (6.4), 4-19	104/465 (22.4), 0-33
BRAF[b]	6/92 (6.5)	4/49 (8.2)	0/29 (0)	0/19 (0)	0/10 (0)	2/14 (14.3)	11/105 (10.5), 0-40	25/287 (8.7), 0-56
ELF3	6/92 (6.5)	5/49 (10.2)	0/29 (0)	0/19 (0)	0/10 (0)	1/14 (7.1)	3/27 (11.1)	10/181 (5.5)

The total number of cases examined was 124. Of the 124 cases, 92 had sufficient quality for the DNA NGS analysis, including 49 MBT, 29 MC, and 14 equivocal MBT/MC cases. In total, 727 genes were evaluated and analyzed by the capture NGS. The DNA NGS analyses focused only on the detection of point mutations and short indels of genes included in the panel. It was not possible to interpret copy number variation data in our formalin-fixed and paraffin-embedded sample set; therefore, amplifications or deletions were not evaluated.

MBT, mucinous borderline tumor; MC, mucinous carcinoma; MC exp, MC with expansile type of invasion; MC inf, MC with infiltrative invasion; mucinous all, MBT, MC, and equivocal between MBT/MC all together; (mut), mutation; NGS, next-generation sequencing; (o/a), sum of overexpression (IHC result 3+) and amplification (IHC result 2+ with amplification detected by fluorescence in situ hybridization).

[a] The percentage reflects the number of cases with homozygous or heterozygous deletion spanning *CDKN2A* or a mutation in *CDKN2A*.

[b] One or 2 cases with 2 alterations in the respective gene.

Another problematic issue lies in the diagnosis of intraepithelial carcinoma in MBT.[6,26] In this study, the interobserver variability in distinguishing between MBTs with and without intraepithelial carcinoma was high but with a poor agreement ($\kappa = 0.075$). However, this finding has currently no clinical consequences with respect to patient management.

Because of these difficulties in diagnosing primary ovarian mucinous tumors purely on their histology, ancillary aids would be quite useful. However, so far, no IHC markers or molecular alterations have been found to facilitate the distinction between MBTs and MCs. This is not quite unexpected because MBTs are considered part of a continuum of progression from benign mucinous tumors to MC.[27]

The spectrum and frequency of pathogenic or likely pathogenic (class 4/5) mutations detected in MBTs and MCs were comparable with the findings of the published data, with some exceptions.[28–48] The differences could be due to different methodological approaches for the detection and classification of deleterious mutations. In our study, the entire coding and splicing areas of genes were analyzed, whereas in most previous studies, only the hotspots were analyzed.

In our study, *TP53* mutations were less frequent in MBTs (43% or 30% after excluding nonhotspot mutations) than in MCs (69% or 65% after excluding nonhotspot mutations), whereas previous literature shows a striking difference of *TP53* mutations between MBTs (average 10%) and MCs (average 56%). Most previous studies that reported a low frequency of *TP53* mutations in MBT originated from the same research group and investigated only hotspot mutations using a less-sensitive and comprehensive technology than the NGS approach used in this study.[32,39,46] Similarly, in 2 other studies, only hotspot mutations were investigated.[37,49] In this study, we found 50 class 4/5 *TP53* mutations (in exons 4-8 and 10; NM_000546), including 6 splice mutations, 5 nonsense mutations, 7 frameshift mutations, and 32 missense variants. Interestingly, nonhotspot frameshift *TP53* mutations were detected in exons 4, 5, 7, 8, or 10 in 6 MBT cases and in 1 MC case, which might be missed by an analysis focused on hotspot variants or analysis focused on exons 5-9 as performed in some previous studies.[32,35]

According to the literature, 0% to 33% of ovarian MCs and 4% to 19% of ovarian MBTs harbor HER2 overexpression or amplification.[28,33–35,37,38,44,50] In this study, HER2 amplification/overexpression was significantly more common in MCs than in MBTs (35.3% of all MCs and 45% of MCs with expansile invasion vs 5.3% of MBTs) and as such may play a role in a differential diagnosis. However, the presence of HER2 overexpression/amplification would no more qualify a mucinous tumor for a diagnosis of MC than the lack thereof would qualify a mucinous tumor for a diagnosis of MBT. Interestingly, MCs with expansile invasion more commonly showed HER2 overexpression/amplification and less commonly *HER2* mutation compared with MCs with infiltrative invasion, although this difference was not statistically significant. Most other studies did not perform the *HER2* mutation analysis, but the limited published data suggest that *HER2* mutations are rare in primary ovarian mucinous tumors. Our results revealed a class 4/5 *HER2* mutation in 5.4% of MBTs and in 6.9% of MCs, underlining the importance of analyzing *HER2* oncogenic mutations, because they are potentially targetable similar to HER2 overexpression/amplification.[50,51] *HER2* mutations and HER2 overexpression/amplification are in most cases mutually exclusive in the same tumor, although there may be some exceptions. One of the 5 cases with *HER2* mutation in this study, an MC with expansile invasion, showed a concurrent *HER2* amplification. Certainly, the high frequency of HER2 overexpression/amplification in MCs harbors the opportunity for targeted therapy in a recurrent or metastatic setting.

Other potentially targetable mutations affected the *KRAS*, *BRAF*, PI3K/AKT (*HER2*, *PIK3CA*, *ARID1A*, and *ELF3*) and the Wnt signaling pathways (*RNF43*) and the DNA damage response and the cell cycle regulation (*TP53*, *CDKN2A*, and *ATM*). No pathogenic mutations in *BRCA1* or *BRCA2* genes were found. We did not find any hypermutated or ultramutated cases (defined as $\geq$10 mut/Mb) in our series of cases. The average TMB in MBTs and MCs was similar (1.8 vs 2.3 mut/Mb, respectively), which is in accordance with the results of other studies.[35] Given that our results showed a significant overlap among the detected molecular aberrations, which were not exclusive to either MBT or MC, we conclude that molecular analysis is not beneficial in this differential diagnosis, as has been reported by other authors.[35,43]

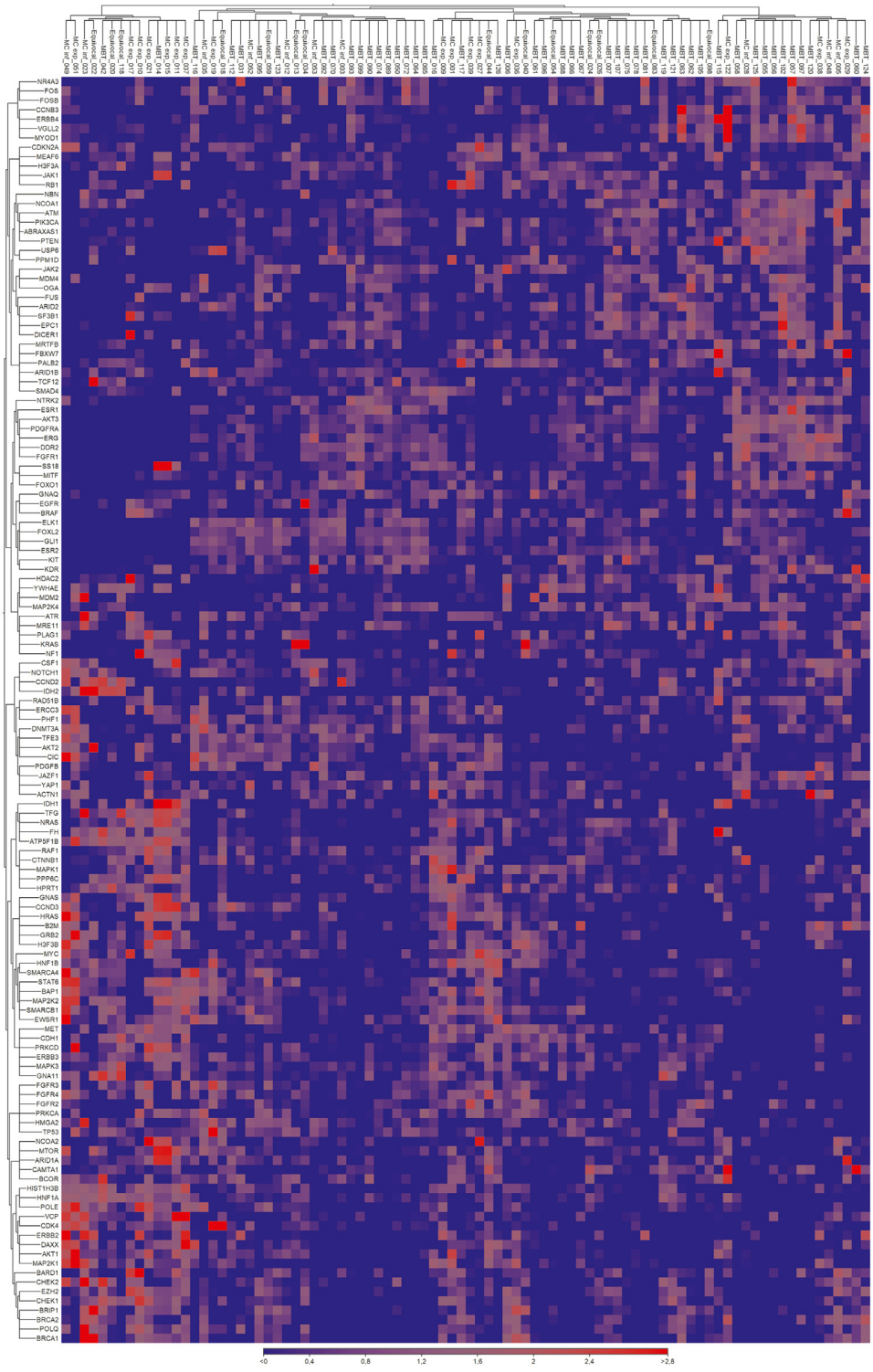

Figure 6.
Expression profiles for 88 mucinous lesions. Expression profiling was performed using the Heat Map for RNA-Seq Analysis module in CLC Genomics Workbench (v21.0.5; Qiagen) with Manhattan distance and Complete linkage settings. Only the samples with sufficient data quality, including 9 mucinous carcinomas with infiltrative invasion (MC inf), 16 MCs with expansile invasion (MC exp), 49 mucinous borderline tumors (MBTs), and 14 equivocal between MBT and MC (equivocal MBT/MC), were visualized in the Heat Map.

The targeted panel RNA-Seq analysis did not show any major stratification between MCs and MBTs. We identified the fusion *TFG::ADGRG7*, which has been reported in many malignancies and even in normal tissues and seems to be frequently involved in tumorigenesis and in cancer predisposition.[52,53] The findings from the fusion and expression profile analyses support the results obtained by IHC and DNA mutation analyses and show that there are no significant differences at the mRNA level between MBTs

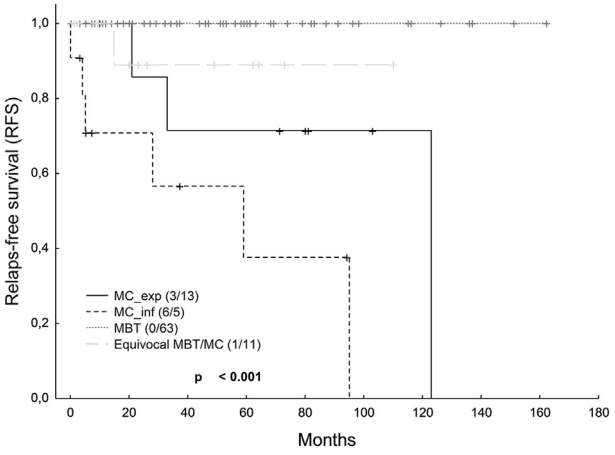

Figure 7.
The relapse-free survival analysis showing the worse survival for mucinous carcinomas (MCs) with infiltrative invasion. Four diagnostic subgroups, 3 groups with consensus diagnoses mucinous borderline tumor (MBT), MC with expansile invasion or MC with infiltrative invasion, and a subgroup of equivocal MBT/MC cases, were compared. The numbers of complete/censored cases are stated in parentheses. Equivocal MBT/MC, cases without consensus diagnoses classifies as equivocal between MBT and MC; MC exp, mucinous carcinomas with expansile invasion; MC inf, mucinous carcinomas with infiltrative invasion.

and MCs. Because our RNA-Seq panel focused on well-described tumor-associated genes, this is limited compared with whole transcriptome-based expression data.

We also addressed the issue of grading of MCs. The grading of MCs is problematic because of the lack of evidence for the prognostic significance of the different grading systems used. Historically, the International Federation of Gynecology and Obstetrics grading system for endometrioid carcinoma was used for MC grading. Recently, the International Collaboration on Cancer Reporting recommended to use the Silverberg system in the ovarian data set but included it as a noncore item.[54] An alternative growth pattern–based system has been proposed, which stratifies MCs based on the amount of infiltrative invasion into 2 categories: low grade (expansile invasion or infiltrative invasion in $\leq$10% of the tumor) and high grade (infiltrative invasion in >10% of the tumor).[14] The results of our questionnaire concerning grading showed that most (80%) but not all participants grade all MCs. The grading systems used differed among the participants, which seems to reflect the absence of a grading system with strong clinical implications. Our participants most commonly used the Silverberg grading, followed by the International Federation of Gynecology and Obstetrics grading and "eyeballing" with no defined system. The use of Silverberg or growth pattern–based grading seems currently the best option, but this area needs further investigation.

We are aware of the limitations of our study. The main limitation is that only a single slide from each case was used for the interobserver variability study and tumor classification. In everyday practice, ovarian mucinous tumors are typically widely sampled, and the design of this study referred to the "worst" areas being chosen. Regarding ancillary markers for a differential diagnosis, especially between MBTs and MCs, the main problem lies in the definition of a diagnostic gold standard. The definition of a diagnostic gold standard is generally problematic in many fields of pathology and particularly for tumor types that represent parts of a biological spectrum with morphologic overlap. Another limitation is that the stratification of the cases into 2 main diagnostic categories (MA and MBT vs MC) was based on agreement between at least 9 of the 14 participants, which was set arbitrarily. Another possible limitation is the use of digital slides. The TMA approach, which harbors the risk of underestimation or overestimation of IHC scoring, despite its widespread use in research, may also be considered a limitation. Finally, our molecular findings should be interpreted with caution because analyses of CNV and epigenetic changes were not performed.

In conclusion, our study underlines the diagnostic difficulties of primary ovarian mucinous tumors, particularly between MBTs and MCs with expansile invasion, even among expert pathologists. The diagnostic difficulties would benefit from the availability of useful ancillary markers for a differential diagnosis, but despite an extensive analysis, we were not able to find molecular alterations with potential diagnostic values. The fact that MBTs and MCs even share similar molecular alterations highlights this diagnostic problem. However, molecular testing can provide the potential for targeted treatment in individual cases, particularly in recurrent or metastatic settings and among others *HER2*, *KRAS*, *PIK3CA*, and *BRAF* may be potential targets.

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Author Contributions

P.D. conceived the study and design and selected slides for scanning. All authors participated in material preparation, data collection, and/or data analyses. R.M. performed the statistical analysis. All authors read and approved the final paper.

Data Availability

The data sets used and/or analyzed during the current study are available from the corresponding author on a reasonable request.

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Declaration of Competing Interest

None reported.

Ethics Approval and Consent to Participate

The study has been approved by the Ethics Committee of General University Hospital in Prague in compliance with the tenets of the Helsinki Declaration (No. 2140/19 S-IV). The Ethics Committee waived the requirement for informed consent because, according to the Czech Law (Act. no. 373/11 and its amendment Act no. 202/17), it is not necessary to obtain informed consent for fully anonymized studies.

Supplementary Material

The online version contains supplementary material available at https://doi.org/10.1016/j.modpat.2022.100040

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