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Title:
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Date:
2013-01-01

Citation:
Anglesio, M. S., Kommoss, S., Tolcher, M. C., Clarke, B., Galletta, L., Porter, H., Damaraju, S., Fereday, S., Winterhoff, B. J., Kalloger, S. E., Senz, J., Yang, W., Steed, H., Allo, G., Ferguson, S., Shaw, P., Teoman, A., Garcia, J. J., Schoolmeester, J. K. ,... McAlpine, J. N. (2013). Molecular characterization of mucinous ovarian tumours supports a stratified treatment approach with HER2 targeting in 19% of carcinomas. Journal of Pathology, 229 (1), pp.111-120. https://doi.org/10.1002/path.4088.

Persistent Link:
https://hdl.handle.net/11343/43903

MOLECULAR CHARACTERIZATION OF MUCINOUS OVARIAN TUMORS SUPPORTS A STRATIFIED TREATMENT APPROACH WITH HER2 TARGETING IN 18% OF CARCINOMAS.

Running title: Molecular Characterization Of Mucinous Ovarian Tumors

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/path.4088.

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No author has any conflict of interest to declare.

Abstract:

Mucinous ovarian carcinomas (MC) typically do not respond to current conventional therapy. We have previously demonstrated amplification of HER2 in 6 of 33 (18.2%) mucinous ovarian carcinomas (MC) and presented anecdotal evidence of response with HER2 targeted treatment in a small series of women with recurrent HER2 amplified (HER2+) MC. Here, we explore HER2 amplification and KRAS mutation status in an independent cohort of 189 MCs and 199 mucinous borderline ovarian tumours (MBOT) and their association to clinicopathologic features. HER2 status was assessed by immunohistochemistry (IHC), FISH and CISH and interpreted per ASCO/CAP guidelines, with intratumoral heterogeneity assessment on full sections, where available. KRAS mutation testing was performed with Sanger sequencing. Stage and grade were associated with recurrence on both univariate and multivariate analysis ($p< 0.001$). Assessment of HER2 status revealed overexpression/amplification of HER2 in 29/154 (18.8%) MC and 11/176 (6.2%) MBOT. There was excellent agreement between IHC, FISH and CISH assessment of HER2 status (perfect concordance of HER2 0 or 1+ IHC with non-amplified status, and 3+ IHC with amplified status). KRAS mutations were seen in 31/71 (43.6%) MC and 26/33 (78.8%) MBOT, and were near mutually exclusive of HER2 amplification. In the 189 MC cases a total of 54 recurrences and 59 deaths (53 of progressive disease) were observed. Within MC either HER2 amplification/overexpression or KRAS mutation was associated with decreased likelihood of disease recurrence ($p=0.019$) or death ($p=0.0041$) when compared to cases with neither feature. Intra-tumoral heterogeneity was noted in 26% of HER2 overexpressing cases. These data support the stratification of MC for the testing of new treatments, with HER2 targeted therapy as a viable option for HER2+ advanced or recurrent disease. Further research is required to delineate the molecular and clinical features of the ~34% of MC cases with neither HER2 amplification nor KRAS mutations.

Key words: breast, cancer, HER2, KRAS, mutation, heterogeneity,

Introduction:

Mucinous ovarian tumors are rare, representing 2-4% of all epithelial ovarian cancers (EOC) [1]. Primary mucinous carcinomas (MC) of the ovary have histological and immunohistochemical features that are more similar to gastric or gastroesophageal cancers than other EOC histotypes and can be challenging to distinguish from tumors of the gastrointestinal (GI) tract that have metastasized to ovary [2-4]. Presentation, distribution of disease, response to therapy, and site of origin of MCs are very different compared to other histologic subtypes of EOC [1,5,6].

The majority of ovarian mucinous tumors are borderline ovarian tumors (MBOT) or stage I mucinous ovarian cancers that do not require additional therapy beyond surgical removal. Prognosis is excellent for these women. In contrast, outcomes in women with metastases beyond the ovaries or with recurrent disease are extremely poor. Response rates of MC to conventional ovarian cancer chemotherapy agents (platinum agent + taxanes) are low [7-12] and efficacy of agents commonly used against gastrointestinal primaries (e.g., oxaliplatin, capecitabine, 5-fluorouracil) in MC are still under investigation but appear unlikely to yield a significant improvement [13,14]. New treatment options are desperately needed for this histologic subtype of EOC.

The study of rare tumors is challenging [15]. Historically, clinical trials in ovarian cancer do not distinguish between histologic subtypes. Where histology is reported, the number of MCs in trials is usually small and/or outcome data insufficient (MC mean time of recurrence extends beyond follow-up for most studies) to assess treatment efficacy. In addition there are few series that have assessed genetic and molecular parameters in mucinous tumors [16-19].

We recently reported a pilot study suggesting HER2 amplification is common in mucinous carcinoma [20], to determine the frequency and clinical significance of HER2 amplification in these cancers we established an international collaboration and collected a cohort of 410 new cases of mucinous tumours. In addition we were able to re-examine some of our previously reported cases [20] adding in depth assessment of intratumoral heterogeneity and KRAS mutational testing. Our objective was to i) validate the frequency of HER2 overexpression and/or amplification in a large number of mucinous carcinoma and

mucinous BOT cases, ii) determine the rate of concordance between IHC, FISH and CISH results and whether standard ASCO/CAP HER2 breast criteria are appropriate in the determination of HER2 status in MC/MBOT, iii) investigate whether or not tumor heterogeneity (intratumoral) of HER2 expression is common in this tumor (as has been observed in gastric carcinoma), iv) examine the frequency of KRAS mutations across HER2 amplified and non-amplified MC/MBOT v) determine the prognostic significance of HER2 and KRAS status in mucinous ovarian tumours, and vi) test for associations of clinical parameters with outcome in mucinous tumours and whether any of these may also be associated with HER2 status.

Methods:

For further experimental details please refer to the supplemental methods

Case Selection and Tissue MicroArray (TMA)

Following Institutional Review Board approval, cases were obtained through the Mayo clinic, Australian Ovarian Cancer Study, the Toronto centers of Princess Margaret Hospital and Toronto General, and the Alberta Cancer Research Biorepository/CBCF-Tumor Bank at Alberta Health Services. Criteria for the diagnosis of primary ovarian MC and MBOT are reviewed in the supplemental methods. Tissue microarrays (TMA) were assembled, with representative sections of primary mucinous ovarian tumors marked by the participating pathologists. For KRAS mutation testing only the Mayo cohort and 19 cases from our own institutional archives and tumor bank ("UBC cohort" previously reported [20]), were available.

Immunohistochemistry

HER2 was evaluated using an anti-HER2 antibody (clone SP3) at a dilution of 1:50 and scored visually according to the ASCO/CAP guidelines[21,22]. Tissue cores that were missing (e.g., all cores missing for duplicate or triplicate samplings), or were otherwise uninterpretable were not included in the analysis.

Fluorescence in situ hybridization

TMA slides were hybridized with probes to LSI® Her-2/neu and CEP® 17 with the PathVysion™ HER-2 DNA Probe Kit and visualized on a Zeiss Axioplan epifluorescent microscope. Analysis of FISH signals was performed both manually and using Metasystems^{TM} automated image acquisition and analysis system, Metafer (Metasystems, Altlussheim, Germany) with scoring per standard guidelines [21].

Dual HER2 Chromogenic Silver in situ Hybridization (CISH)

Slides were prepared and stained with the INFORM HER2 DNA and CHR17 probes and both were visualized on the same slide according to the protocol recommendations in the Ventana SISH Detection kit and ultraView Alkaline Phosphatase Red ISH Detection Kit. *HER2* gene amplification status was evaluated by counting signals in 20 nonoverlapping tumor cells with the highest gene count. Interpretation followed the criteria of the ASCO/CAP guidelines [21,22].

HER2 heterogeneity characterization

Intratumoral heterogeneity was noted on TMAs in some cases, where results differed between cores. In order to assess the degree and frequency of HER2 intratumoral heterogeneity more thoroughly we examined blocks available in our center (cases previously reported) [20] and performed IHC and CISH.

Sequencing

DNA was extracted using the RecoverAll FFPE nucleic acid extraction kit (Ambion). DNA samples were Sanger sequenced for regions encompassing G12/G13 residues of *KRAS* and *NRAS;* Q61 of *NRAS*; V599/V600 of *BRAF*; and A771-A775 *ERBB2 (known activating duplications in the kinase domain)* to screen for activating mutations Products were sequenced on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) and capillary traces were analysed using Mutation Surveyor (SoftGenetics LLC) and visual inspection.

Statistical analysis

Progression free and overall survival was assessed with Kaplan-Meier curves and differences were quantified with the log-rank statistic. Multivariable survival analysis utilizing the Cox Proportional Hazards Model was used to assess the impact of clinicopathologic parameters, immunohistochemical(IHC)

markers, HER2 status, and KRAS mutation status on clinical outcomes. Cohen's kappa statistic was used to assess the level of agreement between HER2 testing modalities. Fisher's exact and Pearson's statistic were used to test for true differences in testing methods, assessing both IHC binary categorization (positive vs. negative on IHC) and 0,1,2,3 IHC score categorization as compared with FISH/CISH.

Results:

Clinicopathologic characteristics

Case contribution from each institution is outlined in Table 1. A secondary review of pathology reports and clinical data was undertaken by our center and cases with mixed histology (e.g., seromucinous BOT), inconsistent clinical data provided (possible pancreatic or gastrointestinal tumor, advanced stage BOT), or completely absent clinical or outcome data were excluded. 199 mucinous borderline ovarian tumors (BOT) of intestinal type and 189 ovarian primary mucinous carcinomas (MC) were carried forward in our analysis (Table 1).

Mean age was 51.6 and 57.8yrs in the MBOT and MC cohorts respectively ($p<0.0001$). 60% of MC were stage I, and 41% were stage IA. Most MCs were confined to the pelvis (73%) but bulky intra-abdominal spread was identified in 17% of cases (3C). Treatment information was available on less than 25% of cases. Surgical debulking rates were high (>90%), consistent with low stage in the majority of cases and/or local pelvic spread. Additional chemotherapy was initiated for $\geq$ stage IC disease in 94% of cases and the majority of these (85%) received platinum/taxane. Table 2 outlines the clinicopathologic parameters of the full cohort; all comparisons were between MBOT and MC ($p<0.0001$ for all comparisons indicated). Follow-up varied considerably in both the MBOT and MC cohorts; however the majority of cases (90%) were between 3-5 years and 4-9 years for MBOT and MC respectively. There were only 6 recurrences out of 199 MBOT cases with a mean time to progression, for these cases, of 3.6 years. All 6 women with MBOT that recurred ultimately died with a mean time to death of 8.7 years. For MC a total of 54 recurrences and 59 deaths out of 189 MC cases were observed (five deaths of unknown cause, with no apparent disease recurrence, and one disease recurrence still alive at last data collection

e.g., 53 documented to have died of disease). Median time to progression and to death was 6.9 years and 13.4 years respectively.

For MC, on univariate analysis, surgical stage was associated with recurrence ($p<0.0001$) and advanced age and stage associated with overall survival ($p=0.0016$ and < 0.0001 respectively). Any woman with evidence of tumour outside of the ovary (i.e. > than stage 1a or 1b) had a markedly worse outcome ($p=<0.0001$). On multivariate analysis both age and stage remained of prognostic significance for overall survival ($p=0.0017$ and < 0.0001 respectively) even with correction for grade, and molecular differences (e.g., HER2 amplification and KRAS mutation status, see below).

HER2 assessment

HER2 overexpression and amplification was three times more common in mucinous carcinomas as compared with mucinous borderline ovarian tumours. For mucinous carcinomas 29/154 or 18.8% of evaluable tumors were HER2 positive in contrast to 11/176 or 6.25% of the evaluable BOT ($p=0.0076$).

There was excellent agreement in HER2 status classification across testing modalities. The kappa statistic, reflecting the level of agreement between *HER2* IHC and CISH, excluding those cases with 2+ HER2 IHC score, was 1.0 (i.e., perfect agreement).). Of 16 cases where IHC score 2+ was recorded, 14 revealed HER2 amplification on CISH and two were negative/no amplification. Similarly, HER2 IHC and FISH showed a high level of concordance, with perfect agreement between FISH and IHC for the negative (IHC score 0 or 1+) or positive (IHC score 3+) groups. Of 8 cases where IHC and FISH were both performed and IHC score was 2+, 6 had amplification of HER2 confirmed with FISH and two did not. There was 100% agreement in FISH and CISH classification of HER2 status where both testing modalities had been performed.

KRAS mutation status

The “UBC cohort” of 19 mucinous tumours (6 MBOT, 13 MC) [20] was subjected to rigorous analysis of activating ras-pathway mutations [17,23]. Samples were tested for by Sanger sequencing for well described activating mutations of KRAS (G12, G13), NRAS (G12, G13, Q61), BRAF (V599, V600), HER2

(kinase domain duplications p.Try772_Ala775dup or p.Ala771_Met774dup), and only *KRAS* mutations were found (Table 3 and details within Supplementary Table).

Having found only, known activating, *KRAS* mutations in the UBC cohort we validated KRAS mutations in a further 151 MC/MBOT tumours (Mayo collection), where a similar pattern was observed (Table 3 and Supplementary Table 1). Of 170 cases subject to *KRAS* mutation testing (151 Mayo, 19 UBC) 29 failed or were not evaluable for either parameter, 14 were assessable for *KRAS* mutation testing only (unknown HER2 amplification status) and 23 had only HER2 amplification status known (failed *KRAS* testing).

Where both HER2-amplification and *KRAS* mutation status was known (n=104) these events were near mutually exclusive. Mucinous tumors could be categorized as follows: 1) HER2 amplification identified, no *KRAS* mutations (n=17; 16 MC,1 MBOT), 2) *KRAS* mutations, no HER2 amplification (n=52; 27 MC, 25 MBOT), 3) both HER2 amplification and *KRAS* mutations (n=5; 4 MC, 1 MBOT), or 4) neither HER2 amplification or *KRAS* mutations (n=30; 24 MC, 6 MBOT) (Figure 1,Table 3, and Supplementary Table 1). The five cases that appeared to have both HER2 amplification and KRAS features are further discussed below (HER2 intratumoral heterogeneity). Although KRAS and HER2 were not completely mutually exclusive there was a statistically significant lack of association of HER2 amplification and KRAS mutations ($p<0.0001$).

KRAS mutations were found in 65 of 118 (55.1%) MC and MBOT. The frequency of KRAS mutation was considerably higher within MBOT (80%) compared to MC (41.0 %) ($p<0.0001$). Within the 104 cases where both HER2 amplification and KRAS mutation status were known the frequency of KRAS mutations were essentially the same: KRAS mutations in 57 of 104 mucinous tumors (54.8%), with mutations present in 31 of 71 MCs (43.6%) and 26 of 33 MBOT (78.8%) (includes cases where both HER2 amplification and KRAS mutations identified) (Table 3).

Intratumoral and molecular heterogeneity

To assess heterogeneity all available blocks of MC and MBOT were sectioned and examined by IHC and CISH. Of 19 cases where whole sections were available and interpretable, intratumoral heterogeneity on IHC scoring was observed in five cases (26%) with distinct areas of HER2 overexpression and complete

absence of HER2 present on the same slide. CISH results on the same 19 cases revealed focal amplification/heterogeneity (Figure 2A-B) in the same five cases (26%), one of which had been classified as negative by FISH in the original series based on TMA assessment alone.

Coexistence of HER2 amplification and KRAS mutations suggests a complex molecular heterogeneity in at least of subset of tumours. These features were observed together in four cases from the Mayo cohort and one case of the UBC cohort. Detailed review of the HER2 IHC, CISH and FISH from these cases revealed significant intratumoral heterogeneity on triplicate or duplicate cores in three of five cases. Classification of the HER2 status of these tumors had been difficult and intratumoral HER2 heterogeneity was noted even within TMA cores. Specifically, IHC scores on triplicate cores for these four cases were as follows (X=not evaluable): 0/2/X, 1/2/2 and X/1/2 and given the equivocal IHC, classification was based on CISH or FISH which also showed heterogeneity on triplicate cores. This left only two of five cases where homogenous HER2 amplification and KRAS mutations were found ("pure double positive"), or 1.9% of total cases (1.4% of MCs) that were evaluable for both events (Supplementary Table 1). Unfortunately none of the noted cases with heterogeneity of HER2 had blocks available for targeted sub-sampling, coring, or microdissection for *KRAS* mutation testing.

HER2, KRAS and prognosis

Cases from all centers with evaluable HER2 data were tested for associations with outcomes. Although there was a trend towards improved progression free survival for cases with demonstrated HER2 overexpression and/or amplification the difference in outcomes was not statistically significant (Figure 3A, Log-rank p=0.1). For overall survival, no association with HER2 status and outcomes was observed (Figure 3B, Log-rank p=0.31). HER2 status was not associated with outcome in MBOT, with the majority of the MBOT cohort censored.

Looking within MCs where both HER2 status and KRAS mutation status were known (n=71) mucinous carcinomas with KRAS mutations or with HER2 amplification were associated with improved PFS (p=0.019) and OS (p<0.0041) (Table 3, Figure 4A-B). Grouping of cases according to KRAS mutation, HER2 amplification, both, or neither was an independent prognostic factor on multivariate analysis after

correction for age, stage and debulking status for both progression free (p=0.0059) and overall survival (p=0.0010).

HER2, KRAS and association with other clinicopathologic parameters

HER 2 positive tumors tended to arise in younger patients than HER2 negative cases in both the BOT and cancer cohorts. For MBOT, mean age with HER2 positive tumors was 49.8 years vs. 51.4 in HER2 negative cases and for MC mean age was 53.5 years with HER2 positive tumors vs. 58 with HER2 negative tumors (p=0.05). Grade and stage were not associated with HER2 status in MBOT nor MC (p=ns).

KRAS mutation status was not associated with age (p=ns) but was associated with earlier stage disease (p=0.01) in mucinous tumors. When assessed within MC only, no association of KRAS mutation with stage was demonstrated.

Discussion:

The study of rare tumors is challenging. Advances require collaborative efforts, enabling the characterization of clinical and molecular parameters in larger cohorts. Knowledge can also be furthered by studying cancers that share morphologic and molecular similarities, regardless of site of origin. In the case of mucinous ovarian carcinomas, there has been much attention given to morphologically similar upper gastrointestinal carcinomas both in considering approach to treatment and in efforts at elucidating the pathogenesis of these tumors. HER2 amplification is common in gastric and gastroesophageal adenocarcinomas (7-15%) [24,25]. In contrast, HER2 positivity has been observed to be low in epithelial ovarian carcinoma (EOC) [26-29] with disappointing results of a large HER2 targeted trial in EOC [27]. These series included few, if any, MCs or had not distinguished histologic subtypes within their cohort. Given our relatively recent appreciation of the distinct molecular profiles of different histologic subtypes of EOC, we anticipated that analysis of MC independent of other histologies might yield quite different results. In 2009 we reported our findings in a small cohort (n=49) of MC and MBOT, and demonstrated HER2 overexpression and amplification in 18% of these tumors. In 2011, Lin et al [30] described HER2 amplification in 4 of 4 MCs examined, with no amplification in the remaining 23 cases of EOC. Now,

through international collaboration we have collected 388 cases of mucinous ovarian tumors (189 MCs and 199 MBOT) and have validated our findings, revealing HER2 amplification in 18% of MCs. We propose that HER2 represents a reasonable target for molecular therapy in HER2 positive metastatic or recurrent MC. We had previously reported favorable outcomes with HER2 targeted therapy (trastuzumab) in a small cohort of cases [20].

Perhaps most importantly, we have characterized different mechanisms of Ras-pathway activation that are exceptionally common in mucinous tumors. The majority of mucinous tumors (71%) have either HER2 amplification or KRAS mutations. Within mucinous carcinomas 66% have either HER2 amplification, KRAS mutation, or both. Similar to other tumor sites, HER2 amplification is near mutually exclusive with KRAS mutations (only co-occurring in ~2-6% of cases tested). Observed heterogeneity of HER2 IHC and copy number (FISH/CISH), and low frequency of “double positive” HER2/KRAS abnormality cases could favor these events occurring later in tumor progression, rather than initiating events. The genomic landscape of mucinous tumors may be more dynamic than that of other epithelial ovarian cancer types, allowing for sub-clonal populations within a tumor to acquire one feature or the other. Further work is clearly warranted to characterize mucinous tumors where neither HER2 amplification nor KRAS mutations were found as well as clarify the molecular heterogeneity within this ovarian carcinoma type.

In terms of the prognostic significance of HER2 or KRAS in MCs patients with either Ras-pathway activating event demonstrate improved progression free and overall survival. This was not apparent when HER2 was assessed and tested for associations with outcomes when KRAS mutation status was not known, likely because of the very favorable outcomes associated with MCs with KRAS mutation confound the HER2 negative cohort. While HER2 cannot be used independently as a prognostic factor to identify those patients with MC who are more likely to recur, it still has potential as a predictive marker, in advanced stage or recurrent disease. There is a precedent for a targetable event in MC being associated with a favorable prognosis: BRAF mutations are common amongst the spectrum of serous borderline and low-grade serous carcinomas and in fact tend to be considerably more common in borderline tumours/non-recurrent serous carcinomas, but rare in advanced stage cases [31], suggesting an

association between BRAF mutations and good prognosis in serous borderline and low-grade serous carcinoma.

The current IHC scoring system of HER2 utilized in breast carcinoma works very well for the assessment of MC. Using this system for MC is attractive as it should be immediately available and familiar in all centers. In the 388 cases of MC reviewed herein there was perfect agreement between IHC, FISH and CISH for IHC negative (0 or 1+) or positive (IHC 3+) cases, indicating that IHC can be used as the primary testing modality. CISH and FISH were also equivalent in evaluation of IHC 2+ cases, however a higher rate of uninterpretable cases was observed with FISH and this technique also requires time-sensitive interpretation (due to fluorescent signal decay), requires a fluorescent microscope, and costs more [32,33]. We therefore recommend IHC testing and, if equivocal (IHC2+), CISH validation should be performed.

Intratumoral heterogeneity was noted to be profound in MC. This has also been observed in gastric carcinomas, and is much higher than observed in breast carcinomas [34-36]. In the case of gastric carcinoma, tumor volume is low and it is easy to recommend thorough examination and sectioning of the entire tumor. However, MC tumor size may exceed 20 cm and complete sectioning is impractical. In addition, HER2 testing only becomes more clinically relevant in cases of metastatic or recurrent disease where additional treatment is needed. Typically disease is confined to the ovary (low stage), requiring surgery only and therefore routine HER2 testing of all MC is not indicated. For recurrent or metastatic disease, it seems reasonable to suggest the assessment of one or more whole sections of carcinoma within the ovarian primary as well as assessment of metastatic or recurrent tumor, when feasible.

Although the 388 cases studied are the largest cohort reported, the limited clinical and outcome information is a study limitation. Also, racial differences in this histology have been reported [37] but that information was not available. Treatments given to women with MC varied with time according to standards for ovarian cancer care and only those with advance stage cancer received adjuvant therapy. Adverse events in long term survivors were likely missed as given the low risk of recurrence in MBOT and early stage MCs many patients are discharged from care/surveillance of cancer centers post operatively. Status was missing with no follow-up information for many cases. The majority (60%) of mucinous

carcinomas in this series were stage I. This is much lower than the often cited 83% stage I MC's described by Seidman et al [38] that included only 6 patients (5 of 6 stage I) but is consistent with the SEER database (Surveillance, Epidemiology and End Results, NCI) describing 55% (of > 4800 mucinous carcinomas) as stage I [39]. There is a risk of underreporting stage I tumors, and missing outcomes as many stage I tumors may never be formally referred to a major center (e.g., receive surgery and surveillance in the community) and could be missed in multi-institutional efforts such as ours.

Like gastric or gastroesophageal tumours, MCs show minimal response to conventional (GI or EOC-based) chemotherapy agents. Clinical trials currently available have taken the approach of i) utilizing agents successful in GI tumors, and/or ii) adding an antiangiogenesis agent to conventional EOC chemotherapy. Accrual for both of these trials has been slow despite concerted efforts at international collaboration. We encourage the participation in histology-specific clinical trials. Novel methods of studying treatment efficacy in rare tumours are needed [40,41]. We believe one option where trials are not available or not feasible may be a monitoring and reporting strategy (**SMART=S**hared Access **M**edicine: an **A**pproach to **R**are **T**umours, www.smartcancerproject.com) wherein shared clinicopathologic data, banked tumor samples, outcome data, and molecular characterization can, further advance knowledge and guide treatment options for women with MC.

These data support the stratification of MC for the testing of new treatments, with HER2 targeted therapy as a viable option for HER2+ advanced or recurrent disease (estimated 18-22% of MCs). We propose a treatment algorithm to consider for this notoriously chemoresistant disease (Figure 5). Although the majority of women with MC will not require additional therapy, for women with widespread metastatic or recurrent mucinous ovarian cancer where treatment is needed, tumors could be tested for HER2, and when positive, targeted therapy can be offered. Concurrent KRAS mutations may predict a lack of response to any anti-EGFR family therapy [42-49]. The very low rate of observed "double positives" (<2% of well characterized cases) in our cohort suggests that KRAS mutation testing may not be required initially. Several "anti-HER2" agents actually target more than one tyrosine kinase inhibitor (i.e., lapatinib inhibits the tyrosine kinase activity of both HER2 and EGFR) and this multi-kinase activity could be advantageous in treatment. Given the success of anti-EGFR inhibitors in KRAS wild type gastrointestinal

tumors combined anti-HER2/anti-EGFR therapy might be particularly attractive. If a HER2 positive tumor was noted to be unresponsive to targeted therapy and/or for cases where no HER2 amplification can be demonstrated KRAS mutation testing could be performed. If wild type KRAS is demonstrated cetuximab therapy (as per colon cancer protocols) is an option. Extrapolation from the colon cancer literature would suggest only half of these patients may respond to cetuximab [43,50] and further characterization of the molecular markers that may predict response in ovarian mucinous carcinomas can be undertaken. In cases with KRAS mutations and no targetable HER2 treatment, there are no novel options available at this time. Conventional therapy (GI or ovarian regimens) or clinical trials can be offered.

Acknowledgements:

The authors wish to thanks the collaborating centers, including the Mayo Clinic (initiated by Dr. Rita Wang), the Australian Ovarian Cancer Study Group (AOCS), the Toronto centers of Princess Margaret Hospital and Toronto General, and the Alberta Cancer Research Biorepository/Canadian Breast Cancer Foundation Tumor Bank at Alberta Health Services (AHS) as well as British Columbia's Ovarian Cancer Research group (OvCaRe) for contributions of biospecimen, clinical and outcome data, as well as intellectual contributions.

In addition the AOCS thanks the study nurses and research assistants for their contribution and all of the women who participated in the study (http://www.aocstudy.org). AOCS is approved by the Human Research Ethics Committees at the Peter MacCallum Cancer Centre, Queensland Institute for Medical Research and all participating hospitals. The Australian Ovarian Cancer Study was supported by the U.S. Army Medical Research and Material Command under DAMD17-01-1-0729, Cancer Council Victoria, Queensland Cancer Fund, Cancer Council New South Wales, Cancer Council South Australia, Cancer Foundation of Western Australia, Cancer Council Tasmania and the National Health and Medical Research Council of Australia (NHMRC).

Contributions:

JNM, CBG, DGH, MSA and SK conceptualized the original study parameters, coordinated sample input, analyzed data, and wrote the final manuscript. JS, WY, and MSA executed *KRAS* mutation screening, HP conducted immunohistochemistry (IHC). JNM, CBG reviewed cases, confirmed histopathology and scored IHC. JBG, JKS, JJG, AT, BJW collected clinical data and contributed case material and information from Mayo cohort. BC, SF, PS collected clinical data and contributed case material and information from the Toronto cohort. HS and SD collected clinical data and contributed case material and information from the Alberta cohort. LG, SF, DDB collected and curated clinical data and contributed case material from the AOCS cohort. SEK and JNM analyzed clinical and outcome data. All authors gave input on the final manuscript.

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Table 1. Case distribution from four contributing centers

	Initial	*Excluded*	*Remaining (n=388)*	
			BOT	*MC*
Alberta	26	3	12	11
AOCS	141	4	101	36
Mayo	162	13	44	105
Toronto	81	2	42	37
Total	410	22	199	189

Table 2. Clinicopathologic parameters within mucinous borderline tumors (MBOTs) and mucinous carcinomas (MCs)

Parameter	*MBOT*	*MC*	*Difference*
Age in years (range)	51.6 (14-88)	57.8 (20-97)	*p<0.0001*
Grade 1 (%)	NA	42%	
Grade 2 (%)	NA	33.7%	
Grade 3 (%)	NA	24.3%	
Stage 1	100% (A=84.2, B=1.4, C=14.4)	60.1% (A=41, B=3.3, C=15.8)	*p<0.0001*
Stage 2		12.6% (A=1.1, B=6, C=5.5)	
Stage3		24 %(A=3.8, B=3.3, C=16.9)	
Stage 4		3.3%	
Follow-up in years (range)	2.9 (1-12.8)	4.4 (1.5-28.6)	*p=0.0001*

Table 3: Summary of HER2 amplification and KRAS mutations

	Combined			UBC			Mayo		
	ALL	MBOT	MC	ALL	MBOT	MC	ALL	MBOT	MC
HER2+, KRAS wt	17 (16.3%)	1 (3%)	16 (22.5%)	5 (26.3%)	0 (0%)	5 (38.5%)	12 (14.1%)	1 (3.7%)	11 (19%)
KRAS mutation, HER2-	52 (50%)	25 (75.8%)	27 (38%)	11 (57.9%)	4 (66.7%)	7 (53.8%)	41 (48.2%)	21 (77.8%)	20 (34.5%)
HER2+, KRAS mutation	5 (4.8%)	1 (3%)	4 (5.6%)	1 (5.3%)	0 (0%)	1 (7.7%)	4 (4.7%)	1 (3.7%)	3 (5.2%)
HER2-, KRAS wt	30 (28.8%)	6 (18.2%)	24 (33.8%)	2 (10.5%)	2 (33.3%)	0 (0%)	28 (32.9%)	4 (14.8%)	24 (41.4%)
Total Cases (n)	104	33	71	19	6	13	85	27	58

Figure Legends:

Figure 1. Distribution of major molecular determinants across mucinous carcinomas, cases with known status of both HER2 and KRAS (n=71) are shown. Grouping, according to known Ras-pathway activating mechanisms is as follows: (1) cases with HER2 amplification and no KRAS mutation (22.5%); (2) cases with KRAS mutation and no HER2 amplification (38.0%); and (3) cases with neither HER2 amplification, nor KRAS mutations. A fourth class with both HER2 amplification and KRAS mutations represent 5.6% (4 cases). Within this latter group significant intratumoral heterogeneity of HER2 amplification was observed in 3/4 cases, overall suggesting the number of "true" overlapping ras-pathway activating features may be much lower.

Figure 2. Intratumoural heterogeneity of HER2 DNA copy-number status in mucinous tumours. (A-B) two separate cases assessed with CISH show clearly distinct regions with high DNA copy number HER2 (black arrow) and normal HER2 copy number (White arrow). The black probe identifies HER2 DNA gene locus and the red probe denotes the control region probe (Chr17 centromeric; CEP17)

Figure 3. HER2 Survival analysis. Kaplan-Meier plot illustrates a trend towards improved progression free survival was observed in the cohort of MCs (n=162) with HER2 amplification/overexpression (Log-rank p=0.10). For overall survival, no association with HER2 status was observed (Log-rank p=0.25). HER2 status was not associated with outcome in MBOT (majority of MBOT cohort is censored, denoted by hash marks – see also Supplemental Table 1).

Figure 4. Survival statistics for major groups of mucinous carcinoma. Kaplan-Meier survival analyses within mucinous carcinomas where both KRAS mutation and HER2 status are known (n=71; see also Figure 1) demonstrates improved PFS and OS in tumors with KRAS mutations and with HER2 amplification. Insufficient data is available to infer outcomes of "double positive" KRAS/HER2 abnormality cases. Absence of both KRAS mutation and HER2 amplification was associated with worse

outcomes compared to either feature being present (PFS p=0.019; OS p=0.0041), suggesting this group represents unique sub-class with an as-yet-undefined molecular background.

Figure 5. Proposed treatment algorithm for primary ovarian mucinous carcinoma. HER2 amplification is present in at least 18.8% of MCs and represents a viable target for patients needing treatment (recurrent or metastatic mucinous carcinoma) where current conventional chemotherapy is largely ineffective. KRAS mutation testing can be performed when HER2 treatment fails or in tumours without HER2 amplification. For KRAS wildtype MC anti-EGFR agents may be considered e.g., cetuximab.

Supplemental Information for On-Line Publication only

Supplemental Methods: Additional experimental details.

Supplemental Table 1: Cohort details for Mayo and UBC samples subjected to HER2 and KRAS screening.

Figure 1

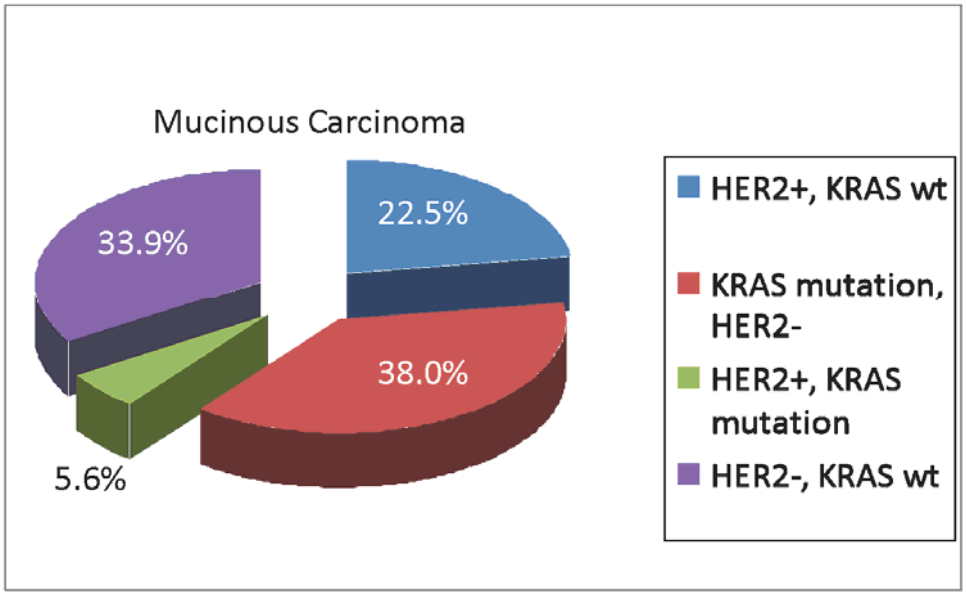

Mucinous Carcinoma
22.5%
38.0%
5.6%
33.9%
HER2+, KRAS wt
KRAS mutation, HER2-
HER2+, KRAS mutation
HER2-, KRAS wt

Figure 2

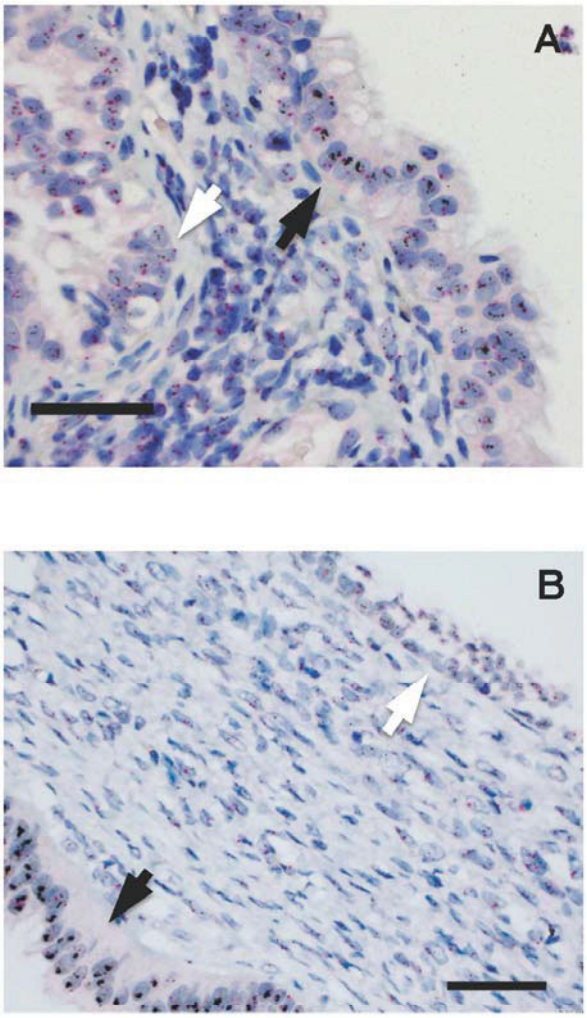


Figure 3

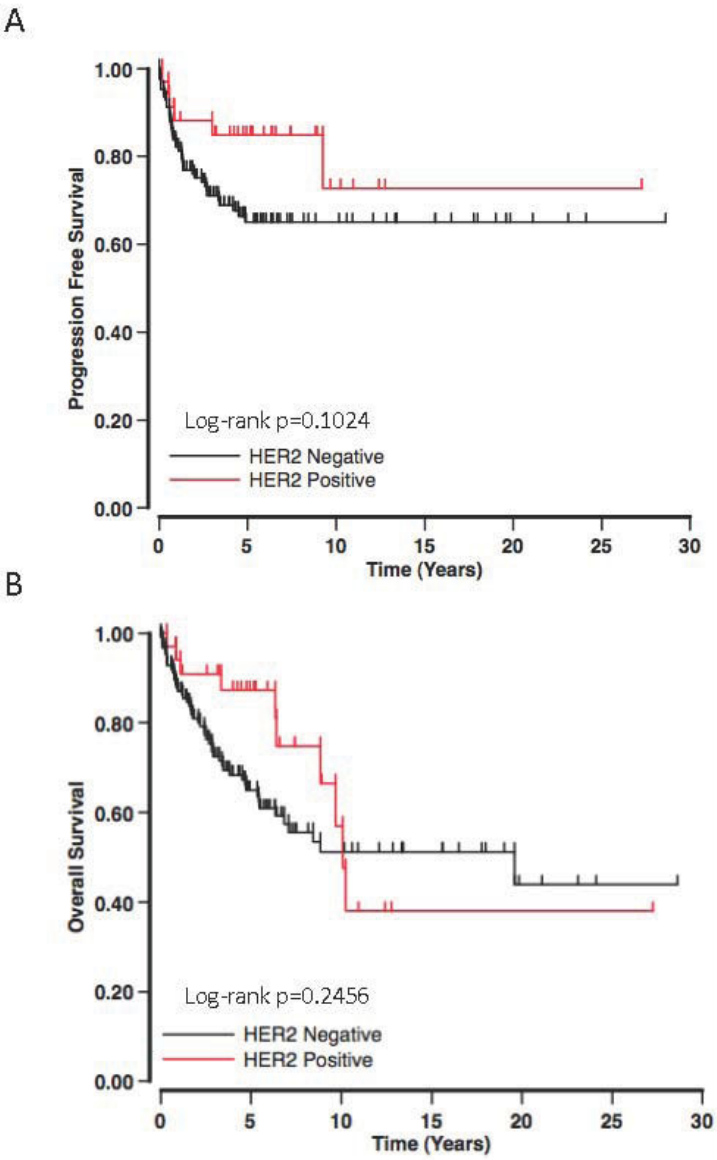

A
1.00
0.80
0.60
0.40
0.20
0.00
Progression Free Survival
Log-rank p=0.1024
HER2 Negative
HER2 Positive
0
5
10
15
20
25
30
Time (Years)
B
1.00
0.80
0.60
0.40
0.20
0.00
Overall Survival
Log-rank p=0.2456
HER2 Negative
HER2 Positive
0
5
10
15
20
25
30
Time (Years)

Figure 4

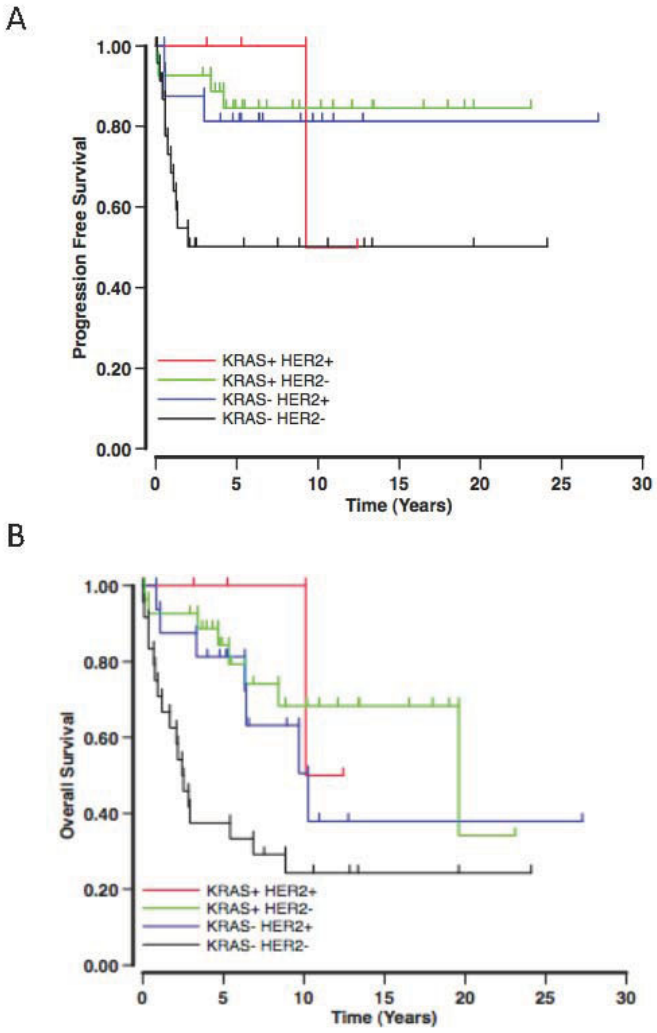
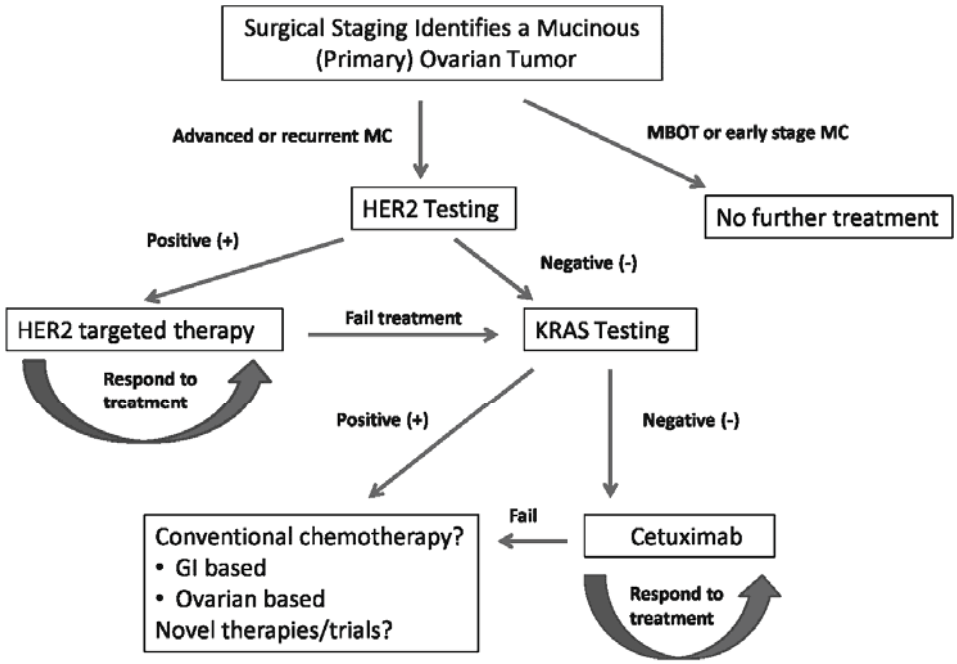

A
1.00
0.80
0.60
0.40
0.20
0.00
Progression Free Survival
KRAS+ HER2+
KRAS+ HER2-
KRAS- HER2+
KRAS- HER2-
0
5
10
15
20
25
30
Time (Years)
B
1.00
0.80
0.60
0.40
0.20
0.00
Overall Survival
KRAS+ HER2+
KRAS+ HER2-
KRAS- HER2+
KRAS- HER2-
0
5
10
15
20
25
30
Time (Years)

Figure 5


Surgical Staging Identifies a Mucinous (Primary) Ovarian Tumor
Advanced or recurrent MC
MBOT or early stage MC
HER2 Testing
No further treatment
Positive (+)
Negative (-)
HER2 targeted therapy
Fail treatment
KRAS Testing
Respond to treatment
Positive (+)
Negative (-)
Fail
Conventional chemotherapy?
• GI based
• Ovarian based
Novel therapies/trials?
Cetuximab
Respond to treatment