Isella C,Mellano A,Galimi F,Petti C,Capussotti L,De Simone M,Bertotti A,Medico E,Muratore A

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MACC1 mRNA levels predict cancer recurrence after resection of colorectal cancer liver metastases.

Claudio Isella, Ph.D.*[1,4], Alfredo Mellano, M.D.*[2], Francesco Galimi, Dr.[3], Consalvo Petti, Ph.D.[1], Lorenzo Capussotti, M.D.[5], Michele De Simone, M.D.[2], Andrea Bertotti, M.D., Ph.D.[3,4], Enzo Medico, M.D., Ph.D.,[§1,4], and Andrea Muratore, M.D.[§2]

*Co-first author; [§]Co-senior author

[1]Laboratory of Oncogenomics, [2]Department of Surgical Oncology, [3]Laboratory of Molecular Pharmacology, Institute for Cancer Research and Treatment, Candiolo, Italy.

[4]Department of Oncological Sciences, University of Torino Medical School, Candiolo, Torino, Italy

[5]Department of HPB and Digestive Surgery, Mauriziano Hospital, Torino, Italy

Corresponding Authors (reprints will not be available from the authors):

Claudio Isella

Institute for Cancer Research and Treatment, S.P. 142, km 3,95 – 10060 Candiolo (TO) Italy

Phone: +39-011-9933520 - Email: claudio.isella@ircc.it

Andrea Muratore

Institute for Cancer Research and Treatment, S.P. 142, km 3,95 – 10060 Candiolo (TO) Italy

Phone: +39-011-9933026 - Email: andrea.muratore@ircc.it

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Running head: *MACC1 and liver-metastatic CRC prognosis*

Mini-Abstract

MACC1 mRNA expression level is a new easily detectable prognostic biomarker in cancer. In this work we demonstrated, for the first time, that MACC1 expression, measured on liver metastasis specimens, is an independent prognostic factor of recurrence after curative resection of colorectal liver metastases.

Structured Abstract

Objective: upon colon cancer metastasectomy in liver, disease outcome is heterogeneous, ranging from indolent to very aggressive, with early recurrence. The aim of this study is to investigate the capability of metastasis associated in colon cancer1 (MACC1) levels measured in liver metastasis specimens to predict further recurrence of the disease.

Methods: gene expression and gene dosage of MACC1, hepatocyte growth factor (HGF) and hepatocyte growth factor receptor (MET) were assessed using quantitative realtime PCR on a cohort of 64 liver metastasis samples from patients with complete follow-up of 36 months and detailed clinical annotation. The most relevant mutations associated to prognosis in colorectal cancer, KRAS and PIK3CA, were assessed on the same specimens with Sanger sequencing.

Results: receiver operating characteristic (ROC) analysis revealed that MACC1 mRNA abundance is a good indicator of metastatic recurrence (AUC=0.65, $p < 0.05$), while no such results were obtained with MET and HGF nor with gene dosage. Generation of MACC1-based risk classes was capable of successfully separating patients into poor and good prognosis subgroups (HR = 5.236, 95% CI = 1.2068-22.715, $p < 0.05$). Also KRAS mutation was significantly associated with higher risk of recurrence (HR = 2.07, 95% CI = 1.048-4.09, $p < 0.05$) Cox regression multivariate analysis supported the independence of

MACC1, but not KRAS, from known prognostic clinical information (Node Size HR = 3.155, 95% CI = 1.4418-6.905, $p < 0.001$, Preoperative CEA HR = 2.359, 95% CI = 1.0203-5.452, $p < 0.05$, MACC1 HR = 7.2739, 95% CI = 1.6584-31.905, $p < 0.01$).

Conclusions: MACC1, a new easily detectable biomarker in cancer, is an independent prognostic factor of recurrence after liver resection of colorectal cancer metastasis.

Introduction

Liver metastases are observed in up to 50% of the more than 1 million patients diagnosed with colorectal cancer worldwide every year. If feasible, resection of hepatic lesions is the only potentially curative therapy, resulting in 3-year survival rates of up to 60%[1, 2]. Nevertheless, tumor recurrence after curative resection remains a major problem, usually occurring within the first 3 years after surgery[3]. The use of perioperative chemotherapy seems to achieve an approximate 8-10% increase in disease-free survival (DFS) rates at 3 years[4-6]. Recently, a european expert panel has recommended that most patients with resectable colorectal liver metastases should receive perioperative chemotherapy[4]. However, despite a slight increase in DFS, chemotherapy also have toxic effects, either systemic or "locoregional" (liver damage) [4, 5].

Clinical prognostic factors of recurrence have been used to select patients to perioperative chemotherapy and to surgery, but they demonstrated a poor predictive value in terms of long term outcome[7]. In an attempt to derive more robust prognostic information, some authors have combined multiple clinical prognostic factors to formulate multiparametric scoring systems. The first scoring system for patients with colorectal liver metastases was introduced in 1996 by Nordlinger et al, subsequently followed in 1999 by Iwatsuki et al and by Fong et al[8-10]. Despite promising predictive value in training datasets, all the proposed

scoring systems demonstrated limited external validation and their clinical utility remains controversial[11, 12].

Indeed, molecular biomarkers are expected to better stratify patients with colorectal liver metastases beyond what the clinical prognostic factors and scoring systems may offer[13]. Among them, the metastasis-associated in colon cancer-1 (MACC1) gene has promising features: it is frequently overexpressed in metastatic colon cancer, and its levels of expression in the primary lesion are associated with poor prognosis[14-18]. These observations have been extended also to other cancer types, including lung adenocarcinomas[17, 19, 20], hepatocellular and gastric carcinomas[21-24], and ovarian tumors[25, 26]. From the biological point of view, the above studies pinpoint a role for MACC1 in promoting tumor cell motility and invasion, which would then lead to locoregional and systemic dissemination of the disease. This hypothesis is supported by mechanistic data demonstrating that MACC1 can promote an invasive growth program driven by the HGF-Met signaling axis[14, 18, 27, 28]. Based on these findings, a robust rationale emerges clearly, implicating MACC1 and possibly the downstream HGF/Met axis, in the progression of primary tumors toward metastases. MACC1 mRNA is expressed both in primary colon cancer and in colon cancer metastases. However, to our knowledge, no studies have analyzed the prognostic impact of MACC1 mRNA expression in tissue specimens of colorectal liver metastases.

Aim of the present study is to determine the prognostic relevance of MACC1, HGF and MET gene dosage and mRNA expression levels on recurrence-free survival in patients undergoing curative liver resection for colorectal liver metastases.

Materials and Methods

Between October 2008 and March 2010, 113 patients underwent curative liver resection for colorectal metastases at the Department of Surgical Oncology of the Institute for the Research and Cure of Cancer (IRCC, Candiolo, Turin, Italy) and at the Hepato-bilio-pancreatic surgical department of the Mauriziano "Umberto I" Hospital (Turin, Italy). Of these 113 patients, 64 who had complete molecular and clinical data represent the object of the present study.

Preoperative work-up and Selection criteria for surgery

Measurement of carcinoembryonic antigen levels (CEA), contrast-enhanced triple-phase computed tomography of the thorax and abdomen CT), and magnetic resonance imaging with mangafodipir trisodium (MRI) were performed routinely for preoperative staging. 18F-fluorodeoxyglucose positron emission tomography was used in selected patients. Response to neoadjuvant chemotherapy was assessed by CT and MRI according to RECIST criteria[29].

The indocyanine green retention test was routinely performed before surgery to assess liver function. Intraoperative ultrasonography was routinely performed.

Patients were considered candidates for liver resection if all liver metastases were technically resectable with curative intent[30]. Presence of extra-hepatic disease amenable to radical surgery was not considered a contraindication to resection.

Neoadjuvant chemotherapy was performed in patients with initially un-resectable hepatic/extra-hepatic metastases. All patients were periodically reviewed by a multidisciplinary team (hepato-biliary surgeon, oncologist, and radiologist). Liver surgery was performed as soon as metastases became technically resectable.

Adjuvant chemotherapy was not performed routinely but was based on performance status, prognostic factors, and on the number and toxicity of neoadjuvant chemotherapy courses.

Follow-up

Patients underwent abdominal ultrasonography and measurement of serum CEA levels every 4 months during the first 2 years and every 6 months thereafter. CT of the chest and abdomen was scheduled yearly or carried out whenever a recurrence was suspected. Disease free survival was evaluated on first metastatic relapse after liver metastasectomy, thus we defined as good prognosis the patients with DFS greater than 36 months.

Analyte extraction

Nucleic acids were isolated from surgically resected colorectal cancer liver metastases and from matched normal liver tissues, following overnight incubation of the fresh specimens in RNAlater (Ambion), quick freezing at −80°C and mechanical fragmentation. Genomic DNA was isolated using the Blood & Cell Culture DNA Midi Kit (Qiagen). Total RNA was extracted using the miRNeasy Mini Kit (Qiagen) and quality checked with an Agilent 2100 Bioanalyzer (Agilent Technologies). DNA and RNA concentrations were quantified using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific).

Quantitative realtime PCR (qPCR)-based MACC1, MET and HGF gene copy number and mRNA expression together with mutational profile for KRAS, BRAF, PIK3CA and NRAS were previously performed on this cohort of patients, for details see[31, 32].

Statistics

All statistical analyses were performed with R-Bioconductor[33]: univariate and multivariate analyses were performed with the Survival package[34], Receiver operating characteristic with ROCR[35]. Significance for ROC curves was evaluated with the Wilcox test.

To statistically evaluate known clinical prognostic indicators in our patients set, we defined cut-off values according to the work of Fong and colleagues[10]. In particular, we considered as poor prognosis indicators the following five parameters: (i) initial disease stage = N+; (ii)

synchronous metastasis, or metastatic recurrence within 12 months after primary resection; (iii) number of metastatic nodes greater than 1; (iv) maximum liver node diameter > 50 mm; (v) preoperative CEA ≥ 200ng/ml. All analyses were censored at three years, so that poor prognosis cases were those with diagnosed recurrence within 36 months.

To classify patients on the basis of MACC1 expression, we calculated the 5th percentile of the MACC1 mRNA qPCR score in the group of patients who has recurrence within three years. Patients with MACC1 score below the threshold were classified as low risk, the remaining patients as high risk. In this way, we considered that the chosen threshold should bring an acceptable five percent of false negatives (cases with recurrence within 36 months classified as low risk). To evaluate the robustness of the threshold, we performed a Montecarlo simulation with 10'000 iterations[36] where, in each iteration, samples were randomly reassorted in good and poor prognosis groups and the threshold was chosen as the 5th percentile of the random poor prognosis group. The distribution of the 10'000 random threshold values was significantly lower than the real threshold, indicating that, overall, true poor prognosis samples have higher MACC1 expression ($p < 0.005$).

Results

Perioperative Clinical and Molecular characteristics

The clinical and molecular characteristics of the 64 patients whose tumor samples were used are described in Table 1. There were 52 males with a mean age of 66.4 years. In more than two thirds of the patients the primary tumor was located in the colon and had regional metastastic lymph nodes. Liver metastases were diagnosed synchronously in 28 (43.8%) patients; in the 36 metachronous patients, the mean interval free of disease was 16 months

(SD = 9). Most of the patients had multiple, small liver metastases. At final pathology analysis, all the patients had a negative resection margin.

Thirty-six patients (56.3%) received neoadjuvant systemic chemotherapy before liver resection. The chemotherapy regimen was oxaliplatin-based in 15 patients and irinotecan-based in the remaining 20 patients; anti-VEGF monoclonal antibodies were added in 21 patients. Forty-three patients (67.2%) received adjuvant systemic chemotherapy, oxaliplatin-based in 20 patients and Irinotecan-based in 12; anti-VEGF monoclonal antibodies were added in 6 patients. Overall 23 patients (20.3%) underwent both neoadjuvant and adjuvant chemotherapy.

Prognostic assessment of known clinical and molecular parameters.

The median follow-up for disease-free patients was 33 months with 55% of the patient free of disease at 3 years. By univariate Cox regression analysis we evaluated prognostic significance of known clinical parameters associated with long-term outcome[11]. The analysis revealed significant association with poor prognosis for “Node Size” (HR = 2.741, 95% CI = 1.27-5.914, $p < 0.05$) and “pre-resection CEA” (HR = 2.9, 95% CI = 1.296-6.489, $p < 0.001$).

The contribution of oncogenic mutations such as KRAS, PIK3CA, BRAF and NRAS in the context of primary disease[37-42] is undisputed; however only recently their possible role has been explored in the context of metastatic disease[43-45]. In our 64-sample set, KRAS mutation was found in 21 cases and PIK3CA mutation was found in 7 cases. KRAS status showed significant association with DFS (HR = 2.07, 95% CI: 1.048-4.09, $p < 0.05$). Only two and three mutated cases were found for, respectively, BRAF and NRAS, which did not allow statistical analysis. Table 2 shows the results for all the evaluated parameters.

Prognostic assessment of MACC1, MET and HGF mRNA and copy number levels

We explored the possible correlation with DFS of MACC1, MET, and HGF mRNA expression or gene copy variation. ROC curve analysis revealed a significant association between MACC1 mRNA expression, measured by quantitative real-time PCR, and DFS which was the most significant among the variables considered in the analysis (area under the curve = 0.65; $p < 0.05$; Fig 1). According to the ROC curve, a very low false negative rate for tumor recurrence is observed in correspondence of low levels of MACC1 (upper right part of the curve). No significant partitioning capability was observed for MACC1, MET and HGF gene copy variation or for MET/HGF expression, with p-values higher than 0.1 (Supp. Fig1).

To test the performance of MACC1 mRNA level as a prognostic classifier, we defined a qRT-PCR threshold ("MACC1 score" > -1.30) at which only 5% of the poor prognosis cases would be misclassified, thereby subdividing patients in "low-MACC1" (good prognosis; 13 samples) and high-MACC1 (poor prognosis; 51 samples). MACC1-based prognostic stratification was found significant by Cox regression analysis (HR = 5.966, 95% CI: 1.426-24.96, $p = 0.0144$) and Kaplan-Meier with log-rank test, which highlighted a one year-longer DFS for low-MACC1 patients (median DFS = 32.63 vs. 20.23 months, $p < 0.01$; Figure 2A). Interestingly, the percentage of relapses within 36 months was substantially lower in low-MACC1 (15.4%) vs. high-MACC1 patients (64.7%; Fischer exact test p-value < 0.005; Figure 2B).

MACC1 is an independent prognostic classifier.

In the sample set analysed, four prognostic parameters were statistically significant in univariate analysis: "MACC1", "KRAS", "preoperative CEA levels" and "Node Size". To evaluate possible dependencies between these parameters, we carried out multivariate Cox regression analysis. The results, shown in Table 3 (Figure 3), confirmed "MACC1" as an

independent predictor of DFS. Interestingly, only two other variables ("Node Size" and "preoperative CEA") remained significant in this analysis.

We then assessed the distribution of all variables considered for multivariate analysis within the high-MACC1 and low-MACC1 subgroups. "Node Size" and "Preop-CEA" values were evenly distributed across MACC1 expression values. Interestingly instead, KRAS mutation was found to be more frequent in high-MACC1 cases (19/51, 37.3%) than in low-MACC1 cases (2/13, 15.4%). Although not statistically significant (Fisher exact test p-value = 0.1912), this correlation between KRAS mutation and high MACC1 may explain the loss of prognostic significance for KRAS mutation in multivariate analysis.

Finally, we tentatively stratified patients taking into account the three parameters found to be independently significant in multivariate analysis: "NodeSize", "Preop-CEA" and MACC1. A score was calculated as cumulative recurrence risk index, ranging therefore from 0, for patients with no positives (lowest risk), to 3, for patients positive to all parameters. Interestingly, as shown in Figure 4, cases with an index of zero had an extremely low recurrence risk, while 7 out of 10 cases with index = 2 had recurrence within one year, None of the patients had a score of 3.

Discussion

MACC1 has been originally identified through genome-wide expression analyses, comparing primary and metastatic colon cancers[18]. Based on these, MACC1 over expression was proposed as an independent prognostic indicator of metastatic dissemination, which correlates with enhanced invasion and aggressiveness of the primary tumors[14-18]. Macc1 is known to promote transcription of the MET gene, thereby activating the HGF/Met axis and promoting tumor cell motility and invasion[18]; recently a more complex regulatory network

involving downregulation of miRNAs targeting both MACC1 -namely miR-143[46]- and MET –namely miR-1[28]- has been implicated in the promotion of colon cancer cell invasion.

However, we previously observed that pharmacological blockade of Met does not abrogate *in vivo* growth of metastatic colorectal cancer, while *in vivo* blockade of MACC1 was fount to inhibits metastatic dissemination in mouse models, suggesting that MACC1 may operate through additional pathways and mechanisms[31, 47]. Based on these findings, a robust rationale emerges clearly, implicating MACC1 in the metastatization progress from primary tumor.

In this paper we investigate, for the first time, the prognostic impact of MACC1 expression in metastatic colorectal cancer after curative liver resection: high MACC1 levels are associated with significantly higher rates of recurrence within 36 months.

The observation that a player involved bona fide in the invasive process of primary tumor is prognostic also within the context of metastatic disease is not obvious, and deserves further discussion. A potential explanation for this apparent paradox is threefold: (i) the increased invasive potential due to MACC1 overexpression could underlie pre-existent and diffused patterns of undetectable micro-metastatic dissemination of the primary lesion, so that surgery of liver metastases only apparently eradicates the disease; (ii) the metastases overexpressing MACC1 could be locally more invasive *per se*, thus increasing the risk of secondary micrometastasization before resection; (iii) beside its pro-invasive properties, MACC1 could also elicit additional effects on growth or survival of cancer cells. Indeed, we and others have proposed that further MACC1-activity mediators could be hypothesized beside Met[31, 49]. MACC1 expression is endowed with a stronger prognostic power than MET mRNA, which would argue against the idea that MET is the sole mediator of MACC1 biological effects, in line with previous findings[16, 18, 26]. Further studies are needed to clarify this issue, which will involve both high-throughput expression analyses and functional

validation experiments to identify and test the biological relevance of other, MET-independent MACC1 targets within the context of CRC progression.

The potential clinical implications of MACC1 as independent prognostic factor of recurrence are based on its ability to identify two classes of recurrence risk among the patients undergone curative liver resection for colorectal metastases. In Europe, perioperative chemotherapy is a common approach even for patients with resectable colorectal metastases. Recently, a panel of experts has recommended that most patients with resectable colorectal liver metastases should receive perioperative chemotherapy[4]. Particularly, systemic chemotherapy after liver resection of colorectal metastasis, is offered to all the patients fit to treatment. However, a pooled analysis of adjuvant studies has showed only non-significant 10% increase in disease-free survival with a grade 3-4 toxicity in about 30% of the treated patients[5]. Overall, the benefit of adjuvant treatment seems extremely limited, which calls for confining its use to high risk patients' subpopulations, thus preventing useless over treatment and associated toxicities. As a side effect, significant treatment-associated expenses could be spared, which as a whole would improve cost-effectiveness of the therapeutic approach[50]. In the present study, low-MACC1, good prognosis patients had a significantly lower recurrence rate (2 out of 13; 15.4%) than high-MACC1, poor prognosis patients (35 out of 51; 68.6%; p=0.01). Moreover, the prognostic power of MACC1 expression seems to be unrelated to adjuvant therapy (HR = 6.5 in patients treated with Ajuvant therapy, HR = 4.3 for patients not threated) so that a relevant contribution of MACC1 levels in predicting treatment efficacy is unlikely.

This reinforces the notion that MACC1 is a pure prognostic indicator that could be exploited to inform rational therapeutic decisions following surgical intervention. If validated in larger cohorts of patients, our results could justify a MACC1-based categorization of patients, to spare good prognosis patients from useless adjuvant treatment. The technique used to

measure MACC1 mRNA levels, i.e. quantitative realtime PCR, is a well established procedure that has successfully been employed for prognostic assessment in various types of cancer, including colorectal cancer[44], which facilitates further assessments and extensive clinical validation. Moreover, the strong independence of MACC1 from other clinical parameters of prognostic value, i.e. “NodeSize” and “Preop-CEA”, holds promise for further integration and risk stratification scores.

Interestingly, the negative prognostic impact of KRAS mutation on liver metastatic colorectal cancer[45] is confirmed in our data. However, its prognostic significance is lost in multivariate analysis, when MACC1 is included. This finding, together with the fact that KRAS mutation is more frequent in high-MACC1 cases, highlights possible cooperation between the two oncogenes and, if further validated in larger cohorts of patients, could increase the interest for MACC1 assessment in clinical practice.

In conclusion, if confirmed, the results presented here could pave the way for the inclusion of MACC1 expression analysis in a multiparametric score (including molecular, clinical and pathological features) that could help the clinician in assigning aggressive, mild or even none adjuvant regimens to resected patients, depending on their relapse risk, even in the context of metastatic diseases. We are planning to validate both the prognostic relevance of MACC1 and its integration with clinical prognostic factors in a larger cohort of patients undergoing curative liver resection of colorectal cancer metastases.

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Table 1. Perioperative clinical and molecular characteristics of the 74 patients

Clinical characteristics			***Number of patients***
Age (years)*			66.4 (8.9)
Sex (Male)			52 (81.3)
CEA§			39.0 (114.4)
Primary tumour	Site		
		Colon	45 (70.9)
		Rectum	19
Primary tumour	N stage#		
		Positive	43 (67.2)
		Negative	21
Liver metastases			
	Synchronous$		28 (43.8)
	Number*		3.7 (4.6)
		Single	28 (43.8)
		Multiple	36
	Size*		33.6 (sd 20.1)
		> 5 cm	10 (15.6)
		≤5 cm	52
		Not Available	1
	Neoadjuvant Chemotherapy		36 (56.8%)
		Oxaliplatin-based	16
		Irinotecan-based	20
		Targeted Therapy	21
	Adjuvant chemotherapy		43 (67.2%)
		Oxaliplatin-based	31
		Irinotecan-based	12
		Targeted Therapy	6

Values in parentheses are percentages unless indicated otherwise. * Values are mean (s.d.) § Serum carcinoembryonic antigen (CEA) levels (ng/ml) before hepatectomy. # N: lymph node status of the primary tumour. $ At diagnosis.

Table 2. Univariate Cox regression models of disease free survival for clinical parameters

Parameter	Hazard Ratio	Lower .95	Upper .95	P.value
N Stage	2.245	0.9779	5.155	0.0565
Early MTS	0.7556	0.3661	1.559	0.448
NodeNumber	1.079	0.548	2.125	0.825
NodeSize	2.741	1.27	5.914	0.0102*
Preop CEA	2.9	1.296	6.489	0.00959**
NeoAdjuvant	1.357	0.6709	2.744	0.396
Adjuvant	0.7693	0.3636	1.628	0.493
KRAS	2.07	1.048	4.09	0.0362*
PIK3CA	2.049	0.7885	5.325	0.141

*p<0.05 ; **p<0.01

Table 3. Multivariate Cox regression models of disease free survival for molecular parameters

Parameter	Hazard Ratio	Lower .95	Upper .95	P.value
NodeSize	3.4392	1.5634	7.566	0.002135**
Preop CEA	15.1490	3.0906	74.255	0.000805***
MACC1	7.2739	1.6584	31.905	0.008525**
KRAS	1.5843	0.7943	3.160	0.191436

**p<0.01; *p<0.05

Figure Legends

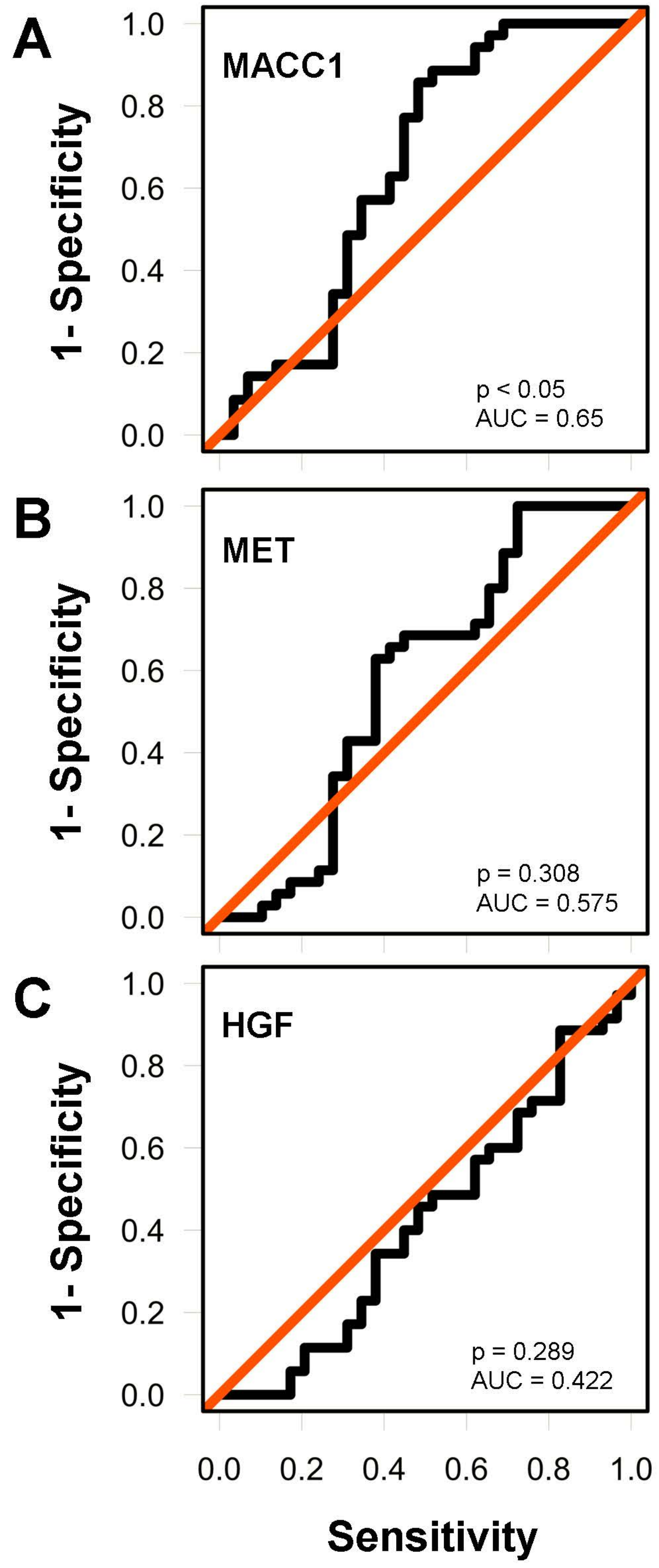
Figure 1. ROC curve analysis for (A) MACC1, (B) MET and (C) HGF mRNA levels

Figure 2. (A) Kaplan-Meier survival analysis of low-MACC1 patients (green line) and high-MACC1 patients (red line). (B) Contingency table displaying the fraction of disease-free or recurred cases within high-MACC1 and low-MACC1 samples.

Figure 3. Forest plot showing HRs and 95% confidence intervals of clinical and molecular parameters, obtained from a multivariate Cox Regression model.

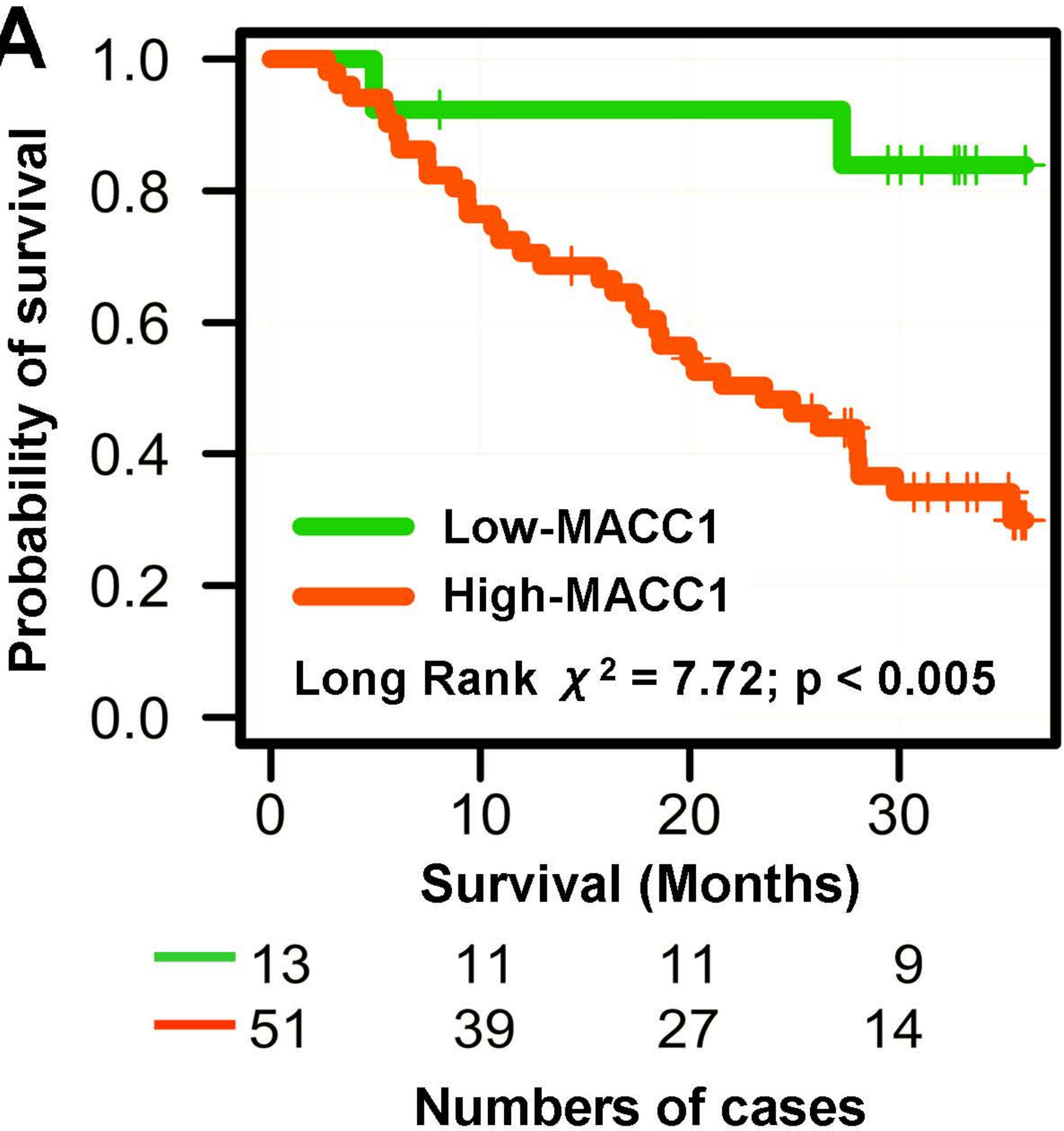
Figure 4. Kaplan-Meier survival analysis of cases subdivided by a combined risk index calculated as the sum of “high-MACC1”, “high-preoperative-CEA” and “Node Size > 5cm”. Patients with no positives (green line) display the lowest recurrence risk. Patients with one positive (blue line) have an intermediate risk. Patients with two positives (red line) have the highest recurrence risk.

Isella et al., Figure 1



Isella et al., Figure 2

A

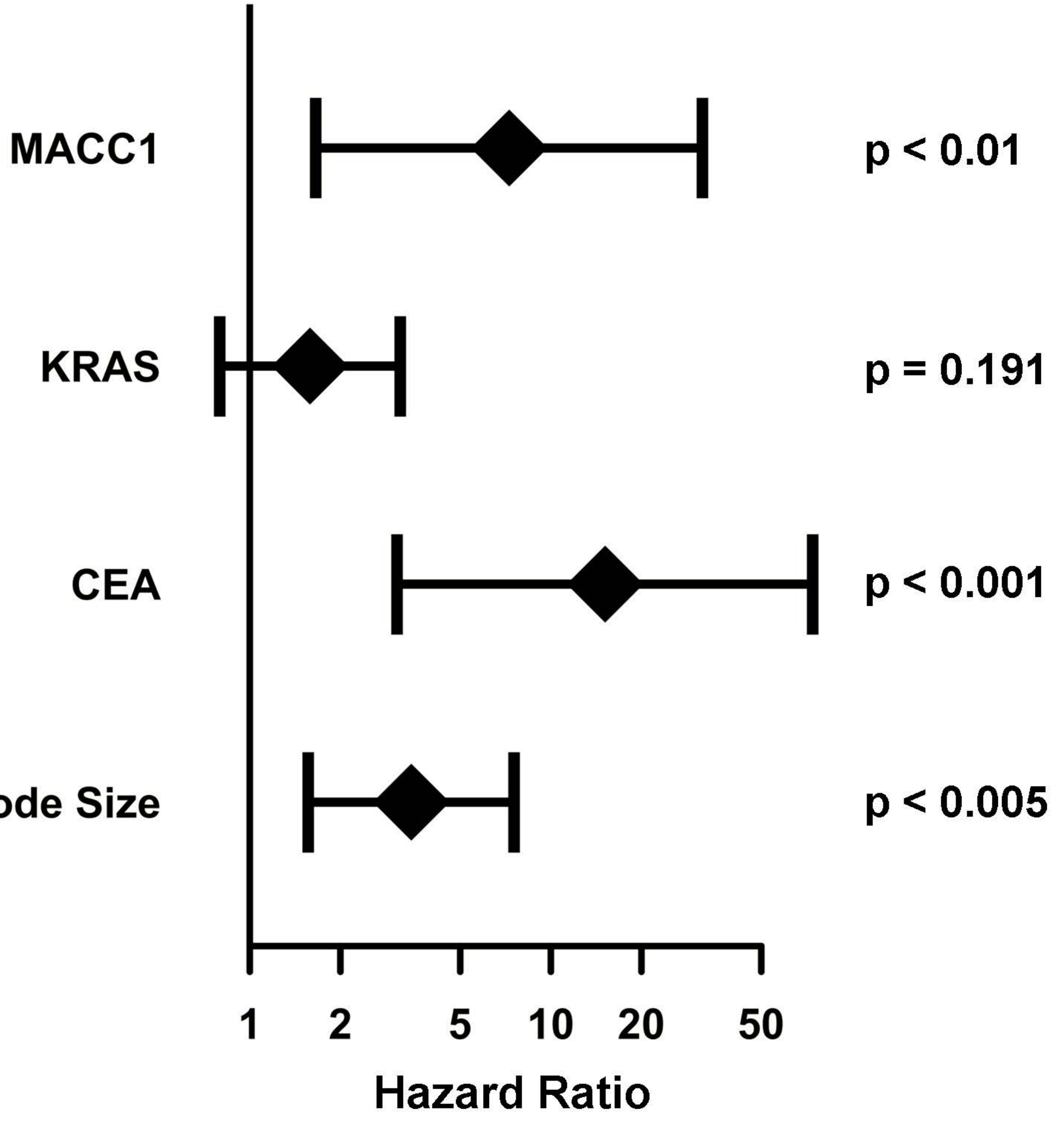


B

MACC1 Status	Disease-free at 36 months	Recurrence within 36 months
Low-MACC1	11	2
High-MACC1	18	33

Fisher Exact test: $p < 0.001$

Isella et al., Figure 3



Isella et al., Figure 4