high. The reported incidence of hepatic artery thrombosis is in the range of 3.1–22 %, and that of hepatic artery stenosis (HAS) is in the range of 4.8–24.6 % [1–7]. The anastomosis procedure using a surgical microscope, first introduced in the 1990s, aimed at better patency and a lesser degree of graft damage in partial liver transplantation [8, 9], and it has since become a standard technique in partial liver transplantation [10, 11]. However, anastomosis using surgical loupes is more popular in some programs because of its advantages over anastomosis using the surgical microscope, such as the time saved for adjusting the operative fields and better focusing in the abdominal cavity [12], with similar surgical outcomes [12–14]. Including comparative studies between the methods using the microscope and surgical loupes [12–14], very few studies have been conducted to investigate the risk factors for the development of hepatic arterial complications.

Doppler ultrasonography (DUS) is the current gold standard for evaluating hepatic arterial thrombosis and stenosis, both intraoperatively and postoperatively. Measurements of the resistive index of the reconstructed hepatic artery, the tardus–parvus waveform, or other useful parameters in a Doppler study have been shown to provide rather accurate diagnosis of HAS [6, 15–19]. Recently, multidetector row computed tomography (MDCT) has been demonstrated to be useful for the evaluation of small-arterial complications, obviating the need for the more invasive angiography, and to also be quite useful for the diagnosis of post-transplant complications [20, 21]. However, there is very little information so far about the usefulness of MDCT in the evaluation of the hepatic artery in liver transplant recipients [21, 22].

In the present study, we attempted to identify the risk factors for anastomotic complications after hepatic artery reconstruction, and examined the role of MDCT in evaluation of the reconstructed hepatic artery in liver transplant recipients.

Patients and methods

A total of 109 adult-to-adult living donor liver transplantations (LDLT), including one re-transplantation, were performed at our institute between 1999 and July 2011; the total of 108 transplant recipients comprised 57 male and 51 female patients, with a mean age of 49.8 ± 12.3 years. The indications for liver transplantation consisted of viral cirrhosis ($n = 67$), cholestatic liver disease ($n = 14$), fulminant liver failure ($n = 8$), and others ($n = 20$). Among the 109 liver transplantations, 7 transplants were ABO-incompatible. The liver grafts consisted of the right lobe in 61 cases, left lobe with or without the caudate in 38 cases, and the right posterior section in 10 cases.

Surgical techniques

Hepatic artery reconstruction was performed under a surgical microscope (OPMI Vario S88, Zeiss, Tokyo, Japan) (MS group, $n = 84$) until January 2009, when we began to adopt surgical loupes (4.5×, Zeiss, Tokyo, Japan) for arterial reconstructions in all cases (SL group, $n = 25$).

The procedures for anastomosis were similar between the MS and SL groups. First, the hepatic artery in both the donor and the recipient was carefully handled, with appropriate preservation of the surrounding connective tissue, so as to avoid skeletonization of the artery. Appropriate alignment of both the length and rotation was determined. End-to-end anastomosis was carried out by interrupted sutures using non-absorbable monofilament 8-0 (polypropylene suture). First, both the dorsal and ventral ends were anastomosed. While the sutures were stretched apart gently by the first assistant, three to four sutures were placed on one side and tied after confirmation of their correct placement through the arterial layers. The other side was then sutured after flipping the artery, keeping the two angle sutures stretched. If there were multiple arteries in the donor liver, all of the arteries were anastomosed, to the extent feasible.

All the surgical procedures were undertaken by two experienced hepatobiliary transplant surgeons.

Immediately after reperfusion of the liver, DUS was performed. Values of the resistive index of the hepatic artery in the liver hilum of less than 0.6 or peak arterial velocity values of less than 15 cm/s at the proximal part of the intrahepatic artery are considered as abnormal, and the anastomosis was always repeated if the intraoperative DUS study was abnormal.

Postoperative anticoagulant therapy

We routinely start standard anticoagulant therapy once the patient's postoperative condition has stabilized. Intravenous administration of heparin sodium is initiated at the dose of 100 U/h when the aPTT (abnormal partial thromboplastin time) is lower than 40 s. When the target aPTT increased to the range of 40–50 s, then the heparin sodium dose was titrated and could be increased to 600 U/h until postoperative day (POD) 28. The anticoagulant therapy was usually discontinued on POD 28; however, if any abnormality was detected on DUS or MDCT, it was continued beyond POD 28. Antiplatelet agents were started for interventional radiology (IVR)-confirmed or DUS-diagnosed HAS until the abnormality improved or resolved. At this point, warfarin was initiated, with the target prothrombin time/international normalized ratio (PT-INR) set at 1.5–2.5, for any portal venous or hepatic venous abnormalities, such as partial thrombosis.

Postoperative evaluation of the hepatic artery anastomosis

Doppler ultrasound was routinely performed twice a day in the immediate postoperative period (until POD 3), with the frequency of the study reduced to once daily until POD 28, and thereafter to once every other day, and finally to once a week during the remaining period of the patient's hospital stay. In addition, diagnostic DUS was also performed anytime in the event of elevation of the serum transaminase levels. The abnormal findings of hepatic artery anastomosis that were considered as warranting hepatic arterial angiography and IVR consisted of the combination of a refractive index (RI) value of less than 0.6 in the DUS study and elevation of the serum AST or ALT (DUS-based criteria).

Interpretation of the CT images was performed by expert radiologists on staff in the hospital. Hepatic arterial complications were classified by these experts into four categories; (1) hepatic arterial thrombosis; (2) hepatic arterial stenosis, defined as anastomotic narrowing of >50 %; (3) suspected or mild hepatic arterial stenosis, defined as anastomotic narrowing of less than 50 %; and (4) normal findings. In April 2005 MDCT was introduced in our hospital; before that date CT had been performed whenever needed for diagnosing suspected hepatic arterial complications. After April 2005, we started prospective dynamic MDCT studies in recipients of liver transplants ($n = 60$), in which dynamic MDCT was performed in the recipients on POD 7, 14, 28, and at postoperative month (POM) 3, 6, and 12.

In contrast to the absolute indication of angiography/ IVR in cases fulfilling DUS-based criteria, abnormal findings such as suspected hepatic arterial stenosis on CT or MDCT alone, in the absence of DUS-based criteria, are not considered clinically significant; therefore IVR was not performed. We defined IVR-confirmed HAS cases as those in which the HAS was confirmed by angiography, and control cases as those not fulfilling the DUS-based criteria.

Risk factor analysis for HAS and evaluation of the role of MDCT

To identify the risk factors for the development of HAS, the following factors were analyzed and compared between the IVR-confirmed HAS group ($n = 6$) and the control group not fulfilling the DUS-based criteria for HAS ($n = 101$): recipient age, preoperative model for end-stage liver disease score (MELD score), donor age, donor arterial diameter, number of anastomoses, anastomosis method (microscope versus surgical loupes), time for anastomosis, graft type (right lobe, left lobe, right lateral sector), ABO incompatibility between donor and recipient, and presence/ absence of acute rejection.

Furthermore, the usefulness of MDCT in the diagnosis of hepatic arterial complications was investigated in the participants of the prospective MDCT study ($n = 60$). The MDCT findings were compared between the IVR-confirmed HAS group ($n = 3$) and the control group not fulfilling the DUS-based criteria for HAS ($n = 57$).

Statistical analysis

Results are expressed as mean $\pm$ standard deviation. Statistical examination of the correlations was based on the Pearson's product-moment correlation. Clinical data of the donors were compared with Student's t test. P values less than 0.05 were considered to indicate statistical significance.

Results

The patient characteristics and summary of the hepatic anastomosis procedure are described in Table 1. The patient background characteristics were similar between the MS group ($n = 85$) and the SL group ($n = 24$). With regard to the graft type, the frequency of right lobe grafts, as compared to left lobe and other grafts, tended to be higher in the MS group than in the SL group, and the graft weight/recipient standard liver volume (GW/SLV) ratio was larger in the MS group than in the SL group ($P = 0.036$ for both). The cold ischemia time was significantly longer in the SL group, while the warm ischemia time was shorter in the SL group than that in the MS group ($P = 0.0001$ and 0.029, respectively). The patient survival curves of the SL and MS groups are shown in Fig. 1. Survival in the SL group was better than in the MS group, although the difference did not reach statistical significance ($P = 0.057$, log rank test).

A single hepatic artery anastomosis was performed in 96 patients (88.1 %), while double anastomoses were performed in 12 cases (11.0 %) and a triple anastomosis was needed in 1 case (0.9 %). The diameter of the main hepatic artery was similar between the MS and SL groups. None of the 109 patients developed the complication of hepatic artery thrombosis, but HAS was diagnosed according to DUS-based criteria in 8 patients (7.3 %); of those eight patients, all of whom went on to have IVR, the diagnosis was confirmed by IVR in 6 (5.5 %). Treatment with percutaneous transarterial balloon dilatation was successful in two cases, whereas failure due to an intimal flap occurred in one case (12.5 %). In another three cases, treatment was not indicated because of technical difficulties, such as meandering proximal artery or arterial spasm (37.5 %). The two cases with HAS diagnosed according to DUS-based criteria alone had trivial stenosis that did not warrant

Table 1 Patient characteristics

	Total (n = 109)	Microscope (MS group) (n = 85)	Surgical loupes (SL group) (n = 24)	P value
Recipient age	49.8 ± 12.3	49.1 ± 12.5	53.1 ± 11.1	0.231
Recipient gender (M/F)	58/51	48/37	10/14	0.199
Indication (viral/cholestatic/fulminant/others)		54/13/9/18	15/5/2/3	0.766
PreOP MELD score	20.7 ± 8.9	20.8 ± 9.0	20.2 ± 8.8	0.865
Donor age	38.1 ± 13.2	38.1 ± 13.1	38.1 ± 13.1	0.953
Donor gender				
Blood type (identical/compatible/incompatible)	76/26/7	57/23/5	19/3/2	0.328
Graft type (right/left/right lateral)	61/38/10	50/25/10	11/13/0	0.036
Graft weight/standard liver volume (%)	48.4 ± 10.2	49.4 ± 10.3	44.1 ± 8.9	0.036
Cold ischemic time (min)	82.1 ± 45.5	73.7 ± 39.6	112.7 ± 48.9	0.0001
Warm ischemic time	43.0 ± 12.0	44.3 ± 12.4	37.8 ± 8.9	0.029
Arterial diameter	2.00 ± 0.76	1.94 ± 0.71	2.18 ± 0.89	0.198
Number of anastomosis				
Single	96 (88.1 %)	76 (89.4 %)	20 (83.3 %)	0.534
Double	12 (11.0 %)	8 (9.4 %)	4 (16.7 %)	
Triple	1 (0.9 %)	1 (1.2 %)		
Hepatic anastomosis time per anastomosis	45.2 ± 19.5	46.4 ± 20.7	38.7 ± 13.1	0.094
Hepatic artery thrombosis	0	0	0	
Hepatic artery stenosis				
Suspected mild stenosis by CT scan image within 12 months	39 (35.8 %)	28 (32.9 %)	11 (45.8 %)	0.245
Angiography performed	8 (7.3 %)	8 (9.4 %)	0	0.118
Angiography confirmed	6 (5.5 %)	6 (7.1 %)	0	0.181

Data are expressed as mean ± standard deviation. P values were calculated by Student's t test. MELD model for end-stage liver disease
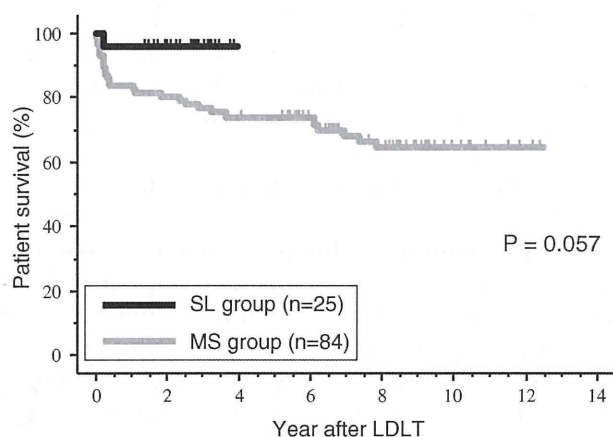
Fig. 1 Patient survival curves after LDLT. The patient survival in the surgical loupe (SL) group was better than that in the microscope (MS) group, although the difference didn't reach statistical significance (P = 0.057, log rank test). Black line SL group (n = 25), Gray line MS group (n = 84)

treatment. Two patients died after IVR, but in neither case was death related to the hepatic arterial complication; both died of bacterial/viral/fungal infections.

In contrast to the patients with DUS-based diagnosis of HAS (n = 8), including those with IVR-confirmed HAS (n = 6), the remaining patients (control group, n = 101) did not develop hepatic artery thrombosis and required no intervention for any hepatic arterial complications throughout the study period.

Risk factor analyses for HAS revealed only ABO incompatibility as being associated with a high risk of development of HAS (P = 0.044). None of the other factors, including arterial diameter and surgical method (microscope or surgical loupes) were found to be significant predictors of HAS (Table 2).

A comparative study of the MS and SL groups showed a tendency in the MS group toward higher frequency of use of right lobe grafts, a shorter cold ischemic time, and longer warm ischemic time, possibly due to its being a chronologically older series. In spite of the similar arterial diameter and number of anastomoses, the duration of performing each anastomosis tended to be shorter in the SL group (38.7 ± 13.1 min) than in the MS group (46.4 ± 20.7 min, P = 0.094). There was no patient among the study subjects

Table 2 Risk factor analyses for HAS

Clinical factors	P value	95 %CI
Surgical method		
Microscope versus surgical loupes	0.975	0.00 to >1000
Age, (years)	0.965	0.94 to 1.07
Gender (M/F)	0.328	0.07 to 2.39
PreOP MELD score	0.403	0.95 to 1.13
Donor age, (years)	0.085	0.82 to 1.01
Arterial diameter, (mm)	0.534	0.47 to 4.34
Anastomotic time, (min)	0.132	0.99 to 1.06
Graft weight/standard liver volume, (%)	0.472	0.00 to 213
Cold ischemia time, (min)	0.268	0.03 to 1.16
Warm ischemia time, (min)	0.416	0.97 to 1.09
Acute cellular rejection	0.983	0.00 to >1000
Graft type (left/right)	0.355	0.32 to 24.9

*CI* confidence interval, *MELD* model for end-stage liver disease

who developed hepatic artery thrombosis, and all of the 6 patients who developed HAS (5.5 %) confirmed by angiography belonged to the MS group.

Multidetector-row CT findings, which were categorized into three types (HAS, suspected/mild HAS, normal), were described for both IVR-confirmed HAS patients ($n = 3$) and the control group not fulfilling the DUS criteria for the diagnosis of HAS ($n = 57$) (Fig. 2). In most cases of IVR-confirmed HAS, the MDCT diagnosis was compatible with IVR-confirmed HAS, whereas a false positive MDCT diagnosis was obtained in a substantial number of cases of the control group. The false positive diagnosis rate of MDCT remained relatively high until 3 months after LDLT (100 % sensitivity and 72.8 % specificity at 3 months), but decreased thereafter until 12 months after LDLT (Fig. 2a, b).

The sensitivity, specificity, and accuracy of MDCT for the diagnosis of HAS are shown in Fig. 2c. The sensitivity was quite high throughout study period, whereas the specificity and accuracy were around 70 % until 6 months after LDLT, improving to over 90 % by 12 months after LDLT.

Discussion

In this study we investigated two different issues related to hepatic arterial anastomosis in LDLT. The first was to identify the risk factors for the development of hepatic arterial anastomotic complications, including a comparison of the surgical methods using either a microscope or surgical loupes for the arterial reconstruction. The second aim of the study was to evaluate the usefulness of MDCT in the diagnosis of hepatic arterial complications.
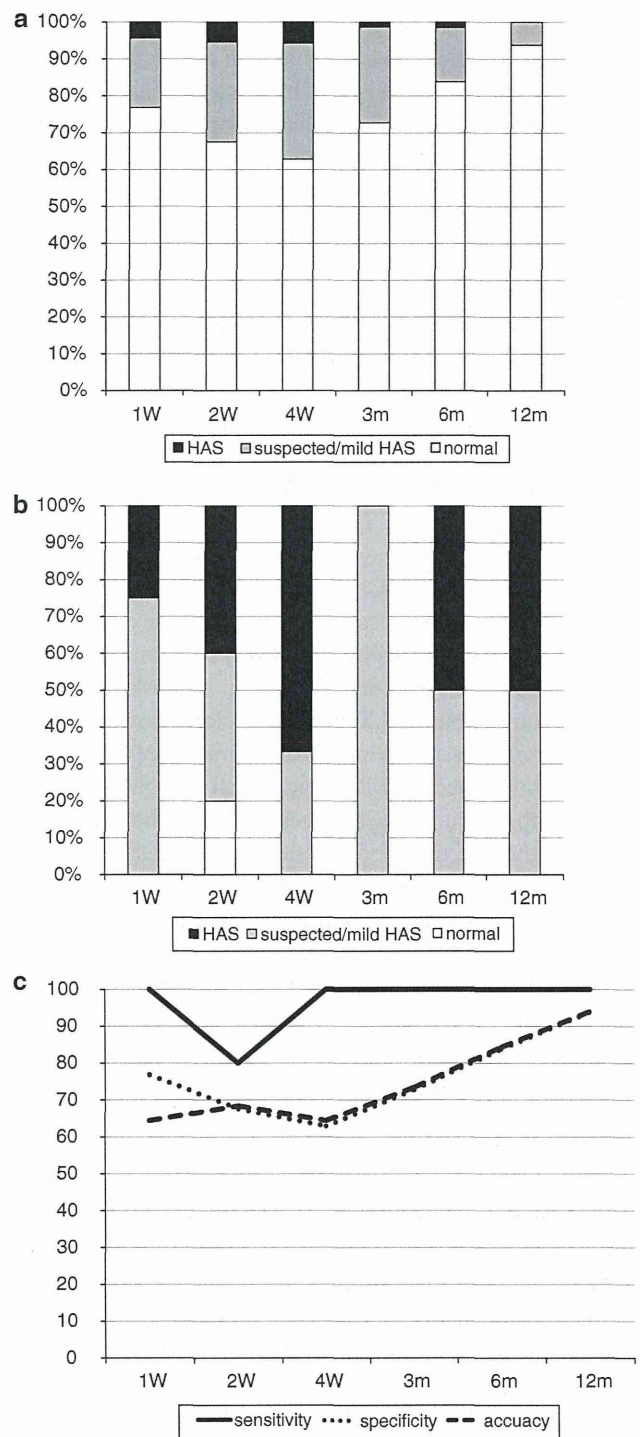
Fig. 2 HAS and MDCT diagnosis. **a** MDCT diagnosis of patients in the control group ($n = 57$), who did not fulfill the DUS-based criteria for the diagnosis of HAS. The false positive rate was relatively high until 3 months after LDLT (100 % sensitivity and 72.8 % specificity at 3 months), but it decreased thereafter up to 12 months after LDLT. **b** MDCT diagnosis in the IVR-confirmed HAS group ($n = 3$). **c** Sensitivity, specificity, and accuracy of MDCT in the diagnosis of HAS. The sensitivity was quite high throughout the study period, whereas the specificity and accuracy were around 70 % until 6 months after LDLT, improving to over 90 % by 12 months after LDLT

The risk factors for hepatic arterial complications after liver transplantation have not yet been clarified, except the anastomosis under a surgical microscope has been considered to be superior, with fewer complications, than that performed with surgical loupes in LDLT [8, 9]. Other studies have reported that continuous end-to-end suturing with a loupe yielded results equivalent to anastomosis under a microscope [23, 24]. In the present study, we found that ABO incompatibility was associated with a high risk of HAS, whereas none of the other factors examined, including the arterial diameter, history of acute cellular rejection, and the anastomosis method (microscope vs. surgical loupes) was found to be associated with the risk of development of HAS. Two (33.3 %) of the six recipients who underwent ABO-incompatible LDLT developed HAS ($P = 0.044$); therefore, this factor was considered a significant risk factor, although this interpretation should be validated with many more cases with ABO-incompatible LDLT. Both recipients survived, with an uneventful postoperative course and without antibody-mediated rejection. The reason underlying the increase in the risk of HAS in ABO-incompatible LDLT is not yet clear; however, there is a possibility of involvement of intimal injury associated with antibody-mediated immunological responses.

A comparative study between our MS and SL groups revealed that the time for hepatic arterial anastomosis was shorter in the SL group than in the MS group. The differences in the graft type, and in the warm and cold ischemic times between the two groups were considered to be mainly related to the chronological differences between the groups, and the influence of these parameters on the anastomosis time was considered to be negligible.

Similarly, the patient survival curve in the SL group was better than that in MS group, and that difference was also considered to be mainly related to the chronological differences between the groups. No case of IVR-confirmed HAS was encountered in the SL group, whereas HAS developed in six patients (7.1 %) in the MS group, although the difference did not reach statistical significance. These results show that the use of surgical loupes with a magnification power of 4.5× yielded at least similar outcomes for the anastomosis, and that the SL procedure was superior to the MS procedure in terms of the time required to perform the anastomosis. Setting up the device is much easier in the case of surgical loupes than in the case of a microscope. Surgical loupes (4.5×) can be safely substituted for a surgical microscope, but the choice should probably be left to the surgeon.

As for the second goal of our study, serial MDCT studies after LDLT showed that the sensitivity of this imaging modality for the detection of HAS was quite excellent within 12 months after LDLT, although the specificity was not optimal; up to 30 % false positive results were obtained, especially in the early post-

transplant period (up to 3 months) after LDLT, whereas MDCT provided diagnosis with a rather high accuracy at 12 months after LDLT. In contrast, DUS-based criteria for HAS, namely, RI >0.6 combined with elevation of the serum AST/ALT, show 100 % sensitivity, 75 % specificity, and 93.6 % accuracy for the diagnosis of HAS during the first 12 months after LDLT. Furthermore, DUS was confirmed as being superior to MDCT for the diagnosis of HAS after LDLT.

Multidetector-row CT was also quite useful in detecting other arterial complications after LDLT [20, 21]. In contrast to a DUS study, MDCT can detect not only abnormalities in the hepatic artery but also abnormalities in other abdominal arteries, the portal vein, the hepatic vein, and the inferior vena cava. We found a superior mesenteric artery aneurysm and stenosis in two patients by MDCT, and both were successfully treated with antiplatelet agents. Blood flow to the liver graft can be evaluated easily by high-resolution MDCT. Therefore, it is worthwhile performing MDCT according to the follow-up schedule described in the present study. However, the rate of false positive diagnosis of HAS was relatively high during the first 3 months after LDLT, and this improved spontaneously over time. These data suggest that the abnormal findings on MDCT not supported by DUS-based criteria represent only a cautionary note for HAS, and that it may be sufficient to monitor the patient's course under therapy with antiplatelet agents, as long as the DUS-based criteria are not fulfilled.

In conclusion, our retrospective study revealed ABO-incompatible LDLT as a risk factor for HAS. Hepatic arterial anastomosis using surgical loupes tended to be time-saving and to yield similar or better results than traditional microscope anastomosis. Also, MDCT was a useful adjunct to a DUS study for the diagnosis of HAS; however, the substantially high rate of false positive diagnosis of HAS should be borne in mind in clinical practice.

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Discovery of colorectal cancer biomarker candidates by membrane proteomic analysis and subsequent verification using selected reaction monitoring and tissue microarray analysis

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Running title: Discovery and validation of membrane protein tumor markers

Abbreviations

iTRAQ: isobaric tag for relative and absolute quantitation

SILAC: stable isotope labeling by amino acids in cell culture

SRM: selected reaction monitoring

MRM: multiple reaction monitoring

PTS: phase-transfer surfactant

SI-peptide: stable isotope-labeled peptide

CID: collision-induced dissociation

HCD: higher energy collision-induced dissociation

IHC: immunohistochemistry

LC-MS/MS: liquid chromatography tandem mass spectrometry

CE: collision energy

LTQ: linear ion trap

FDR: false discovery rate

TMA: tissue microarray

Summary

Recent advances in quantitative proteomic technology have enabled the large-scale

validation of biomarkers. We here performed a quantitative proteomic analysis of

membrane fractions from colorectal cancer tissue to discover biomarker candidates, and

then extensively validated the candidate proteins identified. A total of 5566 proteins

were identified in six tissue samples, each of which was obtained from polyps and

cancer with and without metastasis. GO cellular component analysis predicted that

3087 of these proteins were membrane proteins, while TMHMM algorithm predicted

that 1567 proteins had a transmembrane domain. Differences were observed in the

expression of 159 membrane proteins and 55 extracellular proteins between polyps and

cancer without metastasis, while the expression of 32 membrane proteins and 17

extracellular proteins differed between cancer with and without metastasis. A total of

105 of these biomarker candidates were quantitated using selected (or multiple)

reaction monitoring (SRM/MRM) with stable synthetic isotope-labeled peptides as an

internal control. The results obtained revealed differences in the expression of 69 of

these proteins, and this was subsequently verified in an independent set of patient

samples (polyps (n=10), cancer without metastasis (n=10), cancer with metastasis

(n=10)). Significant differences were observed in the expression of 44 of these proteins,