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Citation	Molecular and Clinical Oncology, 2014, v. 2, p. 817-820
Issued Date	2014
URL	http://hdl.handle.net/10722/207308
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Plasma Bmi1 mRNA as a potential prognostic biomarker for distant metastasis in colorectal cancer patients

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Received January 31, 2014; Accepted April 1, 2014

DOI: 10.3892/mco.2014.321

Abstract. Bmi1 is overexpressed in gastrointestinal cancers, including colorectal cancer (CRC); however, its role as a non-invasive biomarker in CRC has not been established. The aim of this study was to compare the plasma Bmi1 mRNA levels prior to and following curative resection of the primary tumor in CRC patients and to determine their association with the clinicopathological parameters. The plasma Bmi1 mRNA level was measured by quantitative polymerase chain reaction and expressed as cycle threshold value. There was no significant difference between the overall pre- and postoperative plasma Bmi1 mRNA level (31.73±2.63 vs. 31.93±2.88, respectively; P=0.614) in 45 CRC patients. However, when grouped into non-metastatic and metastatic CRC patients, the postoperative Bmi1 transcript level was found to be significantly lower compared to the preoperative level in patients with non-metastatic CRC (32.13±2.677 31.44±2.764, respectively; P=0.041), whereas there was a trend towards a higher postoperative Bmi1 transcript level compared to the preoperative level in the metastatic counterpart (30.85±3.916 vs. 33.27±0.718, respectively; P=0.164). Furthermore, when the patients were categorized into two groups according to their plasma Bmi1 postoperative vs. preoperative level status, we observed that patients without a reduction in the postoperative plasma Bmi1 mRNA levels exhibited a significantly higher rate of distant metastasis following primary resection (P=0.017) and a significantly worse prognosis regarding disease-free survival (P=0.016) when compared to the reduced postoperative plasma Bmi1 level counterparts. In conclusion, plasma Bmi1 mRNA levels may serve as a non-invasive biomarker for monitoring occult metastasis and predicting the development of distant metastasis.

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Key words: Bmi1, colorectal cancer, metastasis

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with >1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 (1). Recent advances in therapeutic strategies and surgical techniques have significantly improved the prognosis of CRC patients with primary disease; however, metastasis is a major concern for CRC patients and physicians. Thirty-five percent of CRC patients have metastatic tumors at the time of diagnosis and 33-50% of the patients without metastases will progress to stage IV during the course of their disease (1,2). Hence, it is necessary to develop prominent biomarkers for detecting the presence of occult metastasis, as well as for predicting the development of future metastasis, which may provide a useful reference for the therapeutic management of CRC patients.

Potential stem cell marker, Bmi1, is a member of the polycomb-repressive complex 1, with a key role in gene silencing through chromatin modifications (3,4). In addition to the proposed role for Bmi1 as a key regulator of cell growth control/senescence mechanisms, accumulating evidence supports the role of Bmi1 in tumorigenesis. Bmi1 is overexpressed in a variety of human cancers and its overexpression was found to be correlated with tumor progression in CRC (5-7) and other gastrointestinal cancers, including esophageal squamous cell carcinoma (8,9), pancreatic cancer (10) and gastric cancer (11), indicating its functional and prognostic role in gastrointestinal cancer patients. The Bmi1 autoantibody in the serum has also been suggested as a minimally invasive biomarker for the prognosis of nasopharyngeal (12), esophageal squamous cell (8) and cervical carcinoma (13). More importantly, circulating Bmi1 mRNA has been detected in the plasma and was correlated with poor prognosis of advanced breast (14) and uterine cervical cancer patients (15). Those studies demonstrated the potential of Bmi1 as a non-invasive surrogate marker for a variety of cancer patients; however, such an application in CRC has not yet been investigated.

In this study, the clinical significance of plasma Bmi1 mRNA in CRC patients was investigated. The pre- and post-operative plasma Bmi1 mRNA levels in CRC patients were determined by quantitative polymerase chain reaction (PCR) and correlated with the clinicopathological parameters, in order to determine whether monitoring of plasma Bmi1 in CRC patients is predictive of the development of distant metastasis.

Materials and methods

Patients and plasma samples. Blood samples were collected from 45 patients who underwent surgical resection of primary CRC during their follow-up at the Department of Surgery, Queen Mary Hospital, The University of Hong Kong, between 2009 and 2013. Among these 45 patients, who presented with no evidence of distant metastasis at the time of the primary resection, 6 developed distant metastasis within 3 years, whereas the remaining patients were recurrence-free at the last follow-up visit to our clinic. The eligibility criteria for this study included pre- and postoperative plasma sample availability for quantitative PCR. Blood was anticoagulated by EDTA and centrifuged at 1,500 g for 10 min. The plasma was collected, divided into aliquots and snap-frozen at -80˚C until use.

This study was approved by the Institutional Review Board of our hospital and the patients provided written informed consent prior to inclusion.

RNA isolation, reverse transcription and quantitative PCR. RNA was isolated from 250 μl of plasma sample using the mirVana™ miRNA isolation kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. For the synthesis of first-strand cDNA, 8 μl RNA was reversed transcribed using PrimeScript™ RT Master mix (Takara Bio, Inc., Shiga, Japan) in accordance with the manufacturer's instructions. Quantitative PCR was performed in a final volume of 15 μl containing 1.5 μl RT transcript, 0.2 μM of each primer, 1X ROX reference dye and 7.5 μl of FastStart Universal SYBR-Green Master (ROX) (Roche Applied Science, Indianapolis, IN, USA). The Bmi1 forward and reverse primer sequences (5'-3') were ATCCCCACCTGATGTGTG and AAAGCCCTGGAACTAATTTG, respectively. Quantitative PCR was performed using the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and at 56˚C for 1 min.

Statistical analysis. The plasma Bmi1 mRNA level was expressed as the cycle threshold (Ct) detected by quantitative PCR using the same threshold value of fluorescent signal intensity. The difference in plasma Bmi1 mRNA level between the two different groups was evaluated by the Student's t-test, whereas the difference between the pre- and postoperative blood levels in the same patient was evaluated by the paired t-test. The differences in recurrence rates among different groups of CRC patients were evaluated by the Fisher's exact test. The disease-free survival was analyzed using the Kaplan-Meier product limit method and the log-rank test. All the statistical analyses were performed with SigmaPlot software, version 10.0 (Systat Software Inc., San Jose, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

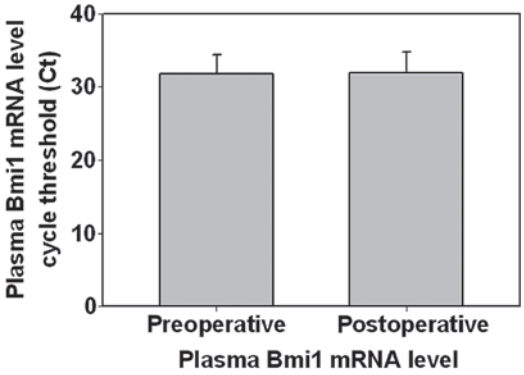

Figure 1. Plasma Bmi1 transcript level prior to and following surgical resection of primary colorectal cancer in 45 patients. There was no significant difference (P=0.614) between the pre- (31.73±2.63) and postoperative (31.93±2.88) Bmi1 transcript levels (expressed as mean cycle threshold (Ct) ± standard deviation).

Results

Correlation of pre- and postoperative plasma Bmi1 mRNA levels with clinicopathological parameters. We first compared the plasma Bmi1 transcript level prior to and following surgical resection of primary colorectal tumor in 45 patients. As shown in Fig. 1, the pre- and postoperative Bmi1 transcript levels (expressed as mean Ct ± standard deviation, i.e., the higher the Ct, the lower the transcript level) were 31.73±2.63 and 31.93±2.88, respectively, without a statistically significant difference between the two levels (P=0.614). We further compared the pre- and postoperative Bmi1 transcript levels in patients with different clinicopathological parameters (Table I). There were no significant changes in pre- and postoperative plasma Bmi1 transcript levels in CRC patients of different age, gender, lymph node status and TNM stage. However, the changes were significantly different between patients with metastatic and non-metastatic CRC. In the 39 non-metastatic CRC patients, the postoperative Bmi1 transcript level was significantly lower compared to the preoperative level (32.13±2.677 vs. 31.44±2.764, respectively; P=0.041). Furthermore, in the 6 CRC patients who developed metastasis, there was a trend for higher postoperative Bmi1 transcript level compared to the preoperative level (30.85±3.916 vs. 33.27±0.718, respectively; P=0.164). These results suggested that an increase in the postoperative Bmi1 level correlated with the development of metastasis in CRC patients.

Due to sampling limitations, the postoperative blood plasma specimens were obtained from CRC patients at different time points following surgery (range, 0.5-25 months; median, 5 months). To determine whether the length of time post-operation affects the plasma Bmi1 levels, we analyzed the association between the length of time post-operation and the difference between post- and preoperative plasma Bmi1 levels in CRC patients. Our results demonstrated that there was no significant correlation (r=-0.0116, P=0.945), suggesting that the length of time post-operation does not affect the results obtained.

Table I. Clinicopathological correlation of plasma Bmi1 mRNA levels in colorectal cancer patients.

Clinico-pathological characteristics	No. of cases[a]	Plasma Bmi1 mRNA level pre- vs. postoperarive (mean Ct ± SD)	P-value
Age (years)			
<65	12	29.82±1.76 vs. 30.07±2.48	0.753
≥65	24	30.36±2.22 vs. 30.18±2.97	0.718
Gender			
Male	22	30.33±2.01 vs. 30.04±3.04	0.657
Female	14	29.96±2.21 vs. 30.29±2.42	0.471
Tumor size (cm)			
<5	15	30.48±2.32 vs. 29.99±3.20	0.523
≥5	9	30.18±2.16 vs. 30.49±2.70	0.589
Lymph node metastasis			
Absent	17	30.04±2.14 vs. 30.57±2.34	0.224
Present	15	30.45±2.27 vs. 29.77±3.49	0.439
TNM stage			
I-II	15	30.68±1.76 vs. 30.90±2.14	0.524
III-IV	12	30.14±2.39 vs. 29.31±3.76	0.457
Distant metastasis			
Absent	32	31.44±2.76 vs. 32.13±2.68	0.041
Present	6	33.27±0.72 vs. 30.85±3.92	0.164

[a]The total number of cases is <45 due to incomplete information. Ct, cycle threshold; SD, standard deviation.

Table II. Plasma Bmi1 mRNA level changes following curative resection of the primary tumor predict development of metastasis.

	Plasma Bmi1 mRNA level	
	Post- >preoperative	Post- <preoperative
Metastatic	5	1
Non-metastatic	11	28
	Fisher's exact test: P=0.017	

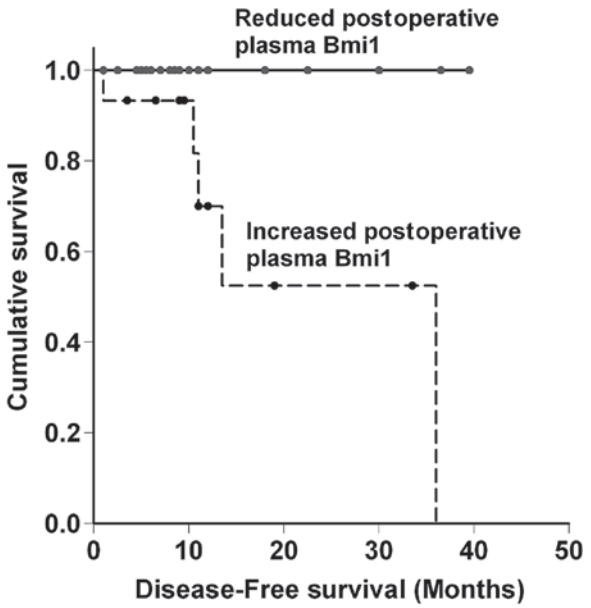

Figure 2. Prognostic significance of changes in post- vs. preoperative Bmi1 levels in colorectal cancer patients. Patients with a decreased postoperative plasma Bmi1 level (n=29) had a significantly better prognosis (P=0.016) for disease-free survival when compared to those with increased plasma postoperative Bmi1 levels (n=16).

Association of higher postoperative plasma Bmi1 mRNA with the development of distant metastasis. We next categorized the 45 patients into two groups according to their plasma Bmi1 post- vs. preoperative level status (Table II). Of the 45 patients, 29 exhibited a reduced postoperative Bmi1 level compared to the preoperative level, whereas 16 displayed increased or unchanged Bmi1 level following resection of the primary tumor. Of the 16 patients who exhibited no reduction in Bmi1 level, 5 developed metastasis within 36 months post-operation. By contrast, only 1 of the 29 patients exhibiting lower postoperative Bmi1 level developed metastasis, suggesting that patients with reduced postoperative Bmi1 levels were less likely to develop metastasis (P=0.017).

Finally, the prognostic significance of the changes in the post- vs. preoperative Bmi1 level in CRC patients was asessed (Fig. 2). The patients with a reduced postoperative Bmi1 level (n=29) had a significantly better prognosis (P=0.016) for disease-free survival when compared to their increased postoperative Bmi1 level counterparts (n=16), further demonstrating the correlation of changes in pre- and postoperative Bmi1 levels with the development of metastasis.

Our results suggested that reduced plasma Bmi1 transcript levels following resection of the primary tumor was a good prognostic factor for CRC patients, whereas patients with an increased Bmi1 level were more likely to develop postoperative metastasis. Therefore, monitoring the changes in the plasma Bmi1 level is a potential prognostic biomarker for the optimal management of CRC patients following curative resection of the primary tumor.

Discussion

Circulating Bmi1 mRNA prior to any treatment has been suggested as a surrogate marker of poor prognosis in patients with breast (14) and uterine cervical cancer (15). In our model, we observed that preoperative circulating Bmi1 mRNA did not correlate with any clinical parameters of CRC patients (data not shown), which may be explained by the relatively small number of patients recruited in this study. However, we demonstrated the significance of monitoring the changes in plasma Bmi1 transcript levels prior to and following surgical resection of the primary tumor in CRC patients, suggesting that monitoring the changes in circulating Bmi1 mRNA levels may be a more accurate prognostic biomarker in CRC patients.

Plasma Bmi1 mRNA levels in cancer patients were found to be significantly higher compared to those in normal subjects (14,15), suggesting that the increased circulating Bmi1 mRNA may originate from the primary tumor and surgical resection of the primary tumor should bring down the levels of such transcripts. Of note, our study did not demonstrate such an effect. We found no significant difference between the overall pre- and postoperative plasma Bmi1 levels in CRC patients. Therefore, we hypothesized that a subset of CRC patients whose Bmi1 levels remained high following primary tumor resection accounted for such findings and we considered that those patients may be at higher risk of developing metastasis, since high Bmi1 levels have been associated with cancer metastasis (15-18). To test our hypothesis, we divided the CRC patients into non-metastatic and metastatic and found that the postoperative circulating Bmi1 mRNA level in non-metastatic patients decreased to a mean value of 0.6-fold of the preoperative level, confirming that resection of the primary tumor removes the source of circulating Bmi1 mRNA. However, metastatic patients exhibited a mean of 5-fold induction in their circulating Bmi1 level postoperatively, which is most likely the result of dissemination of CRC tumor cells that accounted for the development of metastasis.

In the second part of our analysis, we found that monitoring the changes of plasma Bmi1 mRNA prior to and following curative resection was prognostic for the development of future metastasis, suggesting that circulating plasma Bmi1 mRNA may be used as a non-invasive biomarker for predicting and monitoring occult metastasis in CRC patients. We consider this finding to be useful for physicians in the postoperative treatment of CRC patients, such as applying a more aggressive dosage of adjuvant therapy to patients exhibiting no reduction of circulating Bmi1 mRNA after having their primary tumors removed.

Bmi1 mRNA and protein overexpression were previously demonstrated in CRC tumor (5,7) and were positively correlated with tumor stage, invasion and metastasis (6), as well as with poor disease-free and overall survival (6,19), suggesting that Bmi1 is a promising prognostic biomarker in CRC patients. To the best of our knowledge, this study was the first to further demonstrate that the changes in plasma Bmi1 mRNA level prior to and following resection of the primary tumor was prognostic for the development of metastasis, indicating the potential of circulating Bmi1 mRNA levels as a non-invasive, convenient and relatively cost-effective surrogate biomarker for the management of postoperative CRC patients and possibly patients with other types of cancer in which Bmi1 is also overexpressed.

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