c-MYC Amplification in Mucinous Gastric Carcinoma: A Possible Genetic Alteration Leading to Deeply Invasive Tumors

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Abstract. *Background: Patients with mucinous gastric carcinoma (MGC) usually have a poor prognosis, largely due to the advanced stage of disease. In this study, we evaluated the effects of c-MYC amplification on tumor stage and disease-specific survival of 128 patients with MGC and compared the results with those of 302 patients with non-mucinous gastric carcinoma (non-MGC). Patients and Methods: Two-color fluorescence in situ hybridization (FISH) for c-MYC was performed on 430 GC samples. Real-time quantitative polymerase chain reaction (q-PCR) analysis for c-MYC was also performed after tumor microdissection. Results: c-MYC amplification was found in 10.2% of MGCs and 6.0% of non-MGCs. c-MYC amplification was more frequently found in MGCs of higher tumor stage than in MGCs of lower stage (p=0.038). c-MYC amplification in MGC was correlated with greater invasion depth (p=0.007). The mean survival time of patients with c-MYC amplification was shorter than that of patients without c-MYC amplification in MGC. Real-time q-PCR results showed that the calculated c-MYC/GAPDH ratios were higher in c-MYC-amplified MGC than in c-MYC-non-amplified MGC. Conclusion: This study showed that c-MYC amplification in MGC is highly correlated with advanced stage and deeply invasive MGC. This suggests that c-MYC amplification in MGC could be a possible genetic alteration contributing to the frequent presentation of advanced-stage MGC.*

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Key Words: Myc gene, stomach neoplasms, mucinous adenocarcinoma, fluorescence *in situ* hybridization, gene amplification, real-time polymerase chain reaction, survival analysis.

Mucinous gastric carcinoma (MGC) is one of the five main types of gastric carcinoma (GC) according to the World Health Organization (WHO) classification (1). The WHO has defined MGC as a gastric adenocarcinoma with a substantial proportion of extracellular mucin pools, which constitute more than 50% of the tumor volume (1). Patients with MGC frequently have a poor prognosis, largely because the disease presents at an advanced pathological stage (2, 3). It is unclear why MGC aggressively invades the gastric wall and frequently presents at such an advanced tumor stage, and little is known about the role played by genetic alterations in MGC.

c-MYC is an oncogene encoding a transcription factor that plays an essential role in cell proliferation, growth, differentiation, and apoptosis (4). *c-MYC* is broadly expressed during embryogenesis and in adult tissue compartments that possess high proliferative capacity, such as the skin epidermis and the gut (4). Complete loss of *c-MYC* function results in embryonic lethality (5), and in conditional *c-MYC* knockout studies, cells lacking *c-MYC* cease to proliferate and exit the cell cycle (6). Overexpression of *c-MYC* in cultured cells and transgenic animals blocks differentiation and induces neoplastic transformation (5). The knockdown of *c-MYC* can also restrain the growth and proliferation of gastric cancer cells (7). Elevated or de-regulated expression of *c-MYC* has been detected in a wide range of human cancer types and is often associated with aggressive, poorly-differentiated tumors, such as those of the breast, colon, and small-cell lung carcinoma, osteosarcoma, glioblastoma, and malignant melanoma (4). In a wide variety of human cancer types, constitutive overexpression of *c-MYC* largely results from chromosomal translocation and gene amplification (8). Gene

Table I. *Sequences of the oligonucleotide primers and probes used for real-time q-PCR.*

Gene name	Oligonucleotide	Sequence (5'-3')
c-MYC	Forward primer	TGCCTCCCGCTTTGTGT
	Reverse primer	CTGTTGGTGAAGCTAACGTTGAG
	Probe	TCCAGCAGCCTCCCGC
GAPDH	Forward primer	CTCCCCACACACATGCACTTAC
	Reverse primer	CCTAGTCCCAGGGCTTTGATT
	Probe	AAAAGAGCTAGGAAGGACAGGCAACTTGGC

amplification is the most common *c-MYC* aberration in non-hematological malignancies (9). Several studies have shown that c-MYC amplification in GC ranged from 4.0% to 38.1% (10-17). However, only a few studies have shown the clinicopathological implications of c-MYC amplification in GC, while hardly any study has described the implications in MGC. In this study, we compared MGC and non-MGC samples in order to analyze the possible role of *c-MYC* amplification as a genetic alteration causing frequent presentation of MGC at an advanced tumor stage. Moreover, we evaluated the prognostic factors that were correlated with *c-MYC* amplification in MGC. We also assessed the practical usefulness of real-time quantitative polymerase chain reaction (q-PCR) as a substitute for fluorescence *in situ* hybridization (FISH) in the examination of *c-MYC* amplification.

Patients and Methods

Patient samples. A total of 430 GC samples, 128 MGCs and 302 non-MGCs, recorded in the files of the Department of Pathology at Seoul National University College of Medicine were reviewed for clinical information, tissue samples, and FISH analysis of *c-MYC*. This study was approved by the Institutional Review Board of Seoul National University Hospital in accordance with the Declaration of Helsinki.

The patients' age, sex, clinical outcomes, and data regarding tumor size, location, and pathological stage were obtained from medical charts, pathological records, and from the National Statistical Office death statistics. Hematoxylin and eosin-stained glass slides were reviewed to determine the histological types according to Lauren and WHO classifications (1), invasion depth, lymph node metastasis, and the presence of lymphatic invasion and venous invasion. Patients lost during follow-up and those who died due to causes other than gastric cancer were excluded during survival analysis.

Tissue array methods. Core tissue biopsies (diameter, 2 mm) were obtained from individual paraffin-embedded gastric tumors (donor blocks) and arranged in new recipient blocks (tissue microarray blocks) by using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea).

Fluorescence in situ hybridization. Two-color FISH of tumor nuclei was performed using the LSI c-MYC (8q24.12–q24.13) and CEP8 probes (Vysis, Downers Grove, IL, USA). The c-MYC-specific locus probe was labeled in red and the chromosome 8 centromere region in green (Vysis). Briefly, 2-μm sections of de-paraffinized and de-hydrated tissue microarray slides were incubated in 20% sodium bisulfate/2× standard saline citrate at 43˚C for 20 min. After washing in 2× standard saline citrate, the slides were treated with proteinase K at 37˚C for 25 min. Denaturation, hybridization, and post-hybridization washing were carried out according to the manufacturer's instructions. Slides were then counterstained with 4', 6-diamidine-2'-phenylindole dihydrochloride in antifade solution and examined under a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Triple Bandpass Filter Set (Vysis). After counting at least 20 tumor cell nuclei per sample, gene amplification was defined according to the presence of tight gene clusters or when the FISH signal ratio (red signal of c-MYC/green signal of the centromere of chromosome 8) was >2.0.

Microdissection and DNA extraction. DNA was obtained from formalin-fixed, paraffin-embedded surgical sections. The possibility of masking of the genetic abnormalities of tumor cells by normal cells was reduced by selecting tumor areas and microdissecting them with a laser microdissection device (ION LMD II; Jungwoo F&B, Seoul, Korea). DNA was extracted from harvested tumor cells by standard proteinase-K digestion and phenol/chloroform extraction.

Cell lines. Ten human gastric cancer cell lines, namely SNU1, SNU5, SNU16, SNU216, SNU484, SNU601, SNU620, SNU638, SNU668, and SNU719, were used in this study. All cell lines were originally obtained from Korean patients with gastric carcinoma. SNU16 is known to have 4-20 double-minute chromosomes (18). Cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI-1640 (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemicals, St Louis, MO, USA) at 37˚C in an atmosphere with 5% CO_2.

Real-time q-PCR. PCR amplification was performed using the Taqman method in an ABI 7500 instrument (Applied Biosystems, Carlsbad, CA, USA). PCR was performed using 1× premix (TAKARA BIO INC, Otsu, Japan), 100 nmol/l primers, 100 nmol/l probes, and 1 μl of each genomic DNA sample, in a 20-μl final reaction mixture. The primer and probe sequences are described in Table I. The following conditions were used for PCR: Denaturation for 10 s at 95˚C, followed by 40 cycles at 95˚C for 5 s and 60˚C for 1 min. PCR was performed in triplicate. The amplification levels of each sample were normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene.

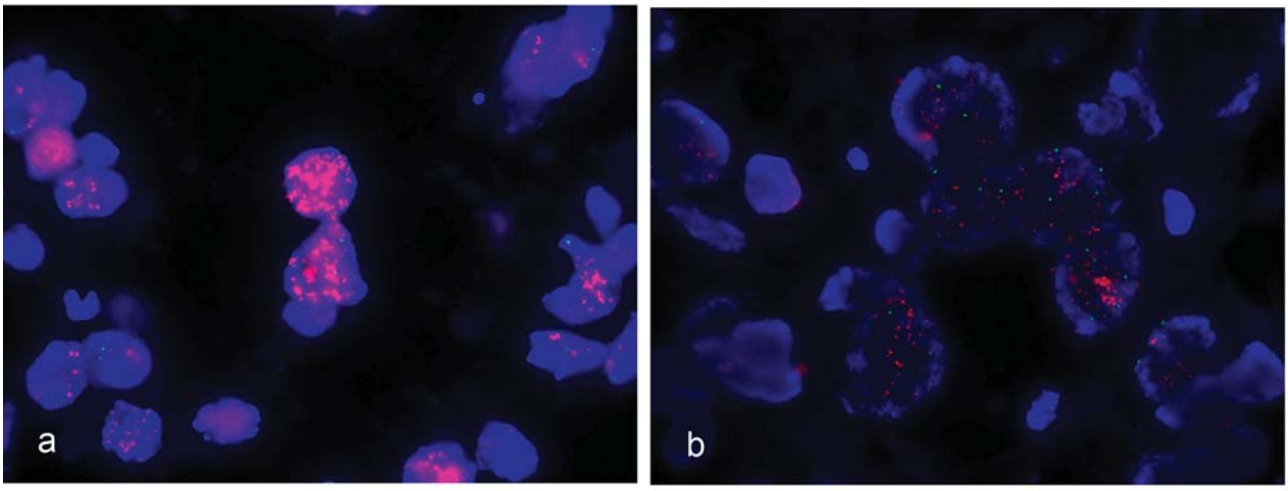

Figure 1. *c-MYC amplification in gastric carcinoma: mucinous gastric carcinoma (a), non-mucinous gastric carcinoma (b).*

Statistical analyses. The chi-square or the Fisher's exact test (two-sided) was used to compare the discontinuous variables, whereas Student's *t*-test was used to compare the continuous variables. Differences were considered to be statistically significant when *p*-values were less than 0.05. Disease-specific survival curves were drawn using the Kaplan–Meier product-limit method, and the significance of the differences between survival curves was determined using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards model. All statistical analyses were conducted using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA).

Table II. *Frequency of c-MYC amplification by fluorescence in situ hybridization in mucinous gastric carcinoma (MGC) and non-mucinous gastric carcinoma (non-MGC) (p=0.153).*

	c-MYC		No. of cases
	No amplification	Amplification	
MGC	115 (89.8%)	13 (10.2%)	128
Non-MGC	284 (94.0%)	18 (6.0%)	302

Results

Frequency of c-MYC amplification in MGC and non-MGC. *c-MYC* amplification was found in 13 (10.2%) of the 128 MGC samples (Figure 1, Table II) and in 18 (6.0%) of the 302 non-MGC samples. Although *c-MYC* amplification was more frequently found in MGC than in non-MGC, there was no statistical difference in the frequency (p=0.153).

Effects of c-MYC amplification on tumor stage and invasion depth in MGC. *c-MYC* amplification was more frequently found in MGC of higher tumor stages (stage III or IV) than in lower tumor stages (stage I or II) (p=0.038) (Table III). Depth of invasion was the tumor stage factor that was most significantly correlated with *c-MYC* amplification (p=0.007). Lymph node and distant metastasis were not correlated with *c-MYC* amplification in MGC. Conversely, in non-MGC, there was no significant difference in the depth of invasion (p=0.106). Lymph node metastasis (p=0.211), distant metastasis (p=0.397), and tumor stage (p=0.075) were not significantly correlated with *c-MYC* amplification in non-MGC, although tumor stage had a borderline significance.

Among the 13 MGC cases with *c-MYC* amplification, tumors in 11 had invaded the serosa (Table IV). All cases of MGC with *c-MYC* amplification, except one, had lymph node metastasis. However, lymph node metastasis and distant metastasis were not statistically correlated with *c-MYC* amplification. None of the MGCs with *c-MYC* amplification was located in the cardia.

Survival outcomes according to c-MYC amplification. The mean survival time of patients with *c-MYC* amplification (39 months) was shorter than that of patients without *c-MYC* amplification (71 months) in MGC. However, there was no statistical difference in the disease-specific survival between patients with *c-MYC*-amplified MGC and those with *c-MYC*-non-amplified MGC (Figure 2a). Patients with *c-MYC*-amplified non-MGC had a poorer disease-specific survival than those without *c-MYC* amplification (p=0.0343) (Figure 2b).

c-MYC amplification measured by real-time q-PCR and its correlation with FISH data. Real-time q-PCR was performed for the 10 gastric carcinoma cell lines. The highest *c-MYC*

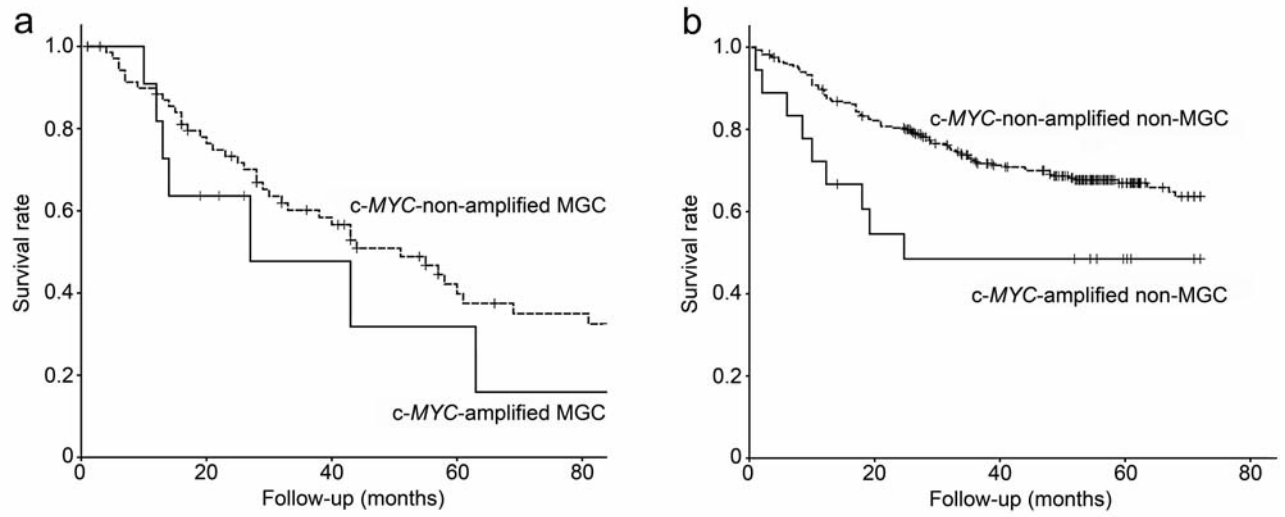

Figure 2. *a: Disease-specific survival curves of patients with c-MYC-amplified and those with non-amplified mucinous gastric carcinoma (MGC) (p=0.3206). b: Disease-specific survival curves of patients with c-MYC-amplified and those with non-amplified non-mucinous gastric carcinoma (non-MGC) (p=0.0343).*

Table III. *Correlation between c-MYC amplification and clinicopathological features in mucinous gastric carcinoma.*

	c-MYC amplification		*p*-Value
	Negative	Positive	
Age (mean, years)	56.3	57.2	0.819
Gender			0.731
Male	87 (75.7%)	11 (84.6%)	
Female	28 (24.3%)	2 (15.4%)	
Stage			0.038
Low (I, II)	54 (47.0%)	2 (15.4%)	
High (III, IV)	61 (53.0%)	11 (84.6%)	
Invasion depth			0.007
pT1, pT2	65 (56.5%)	2 (15.4%)	
pT3, pT4	50 (43.5%)	11 (84.6%)	
LN metastasis			0.298
Negative	26 (22.6%)	1 (7.7%)	
Positive	89 (77.4%)	12 (92.3%)	
Distant metastasis			0.208
Negative	102 (88.7%)	10 (76.9%)	
Positive	13 (11.3%)	3 (23.1%)	
Tumor size (mean, cm)	6.818	7.515	0.549
Lauren classification			0.319
Intestinal	36 (31.3%)	2 (15.4%)	
Diffuse	74 (64.3%)	11 (84.6%)	
Mixed	5 (4.3%)	0 (0.0%)	

LN: Lymph node.

gene expression was observed in SNU16, as expected. Real-time q-PCR was performed in all 13 cases of *c-MYC*-amplified MGCs and in 16 randomly selected tissue samples of *c-MYC*-non-amplified MGCs. The calculated *c-MYC/GAPDH* ratio was highly correlated with *c-MYC* amplification by FISH (p=0.048) (Table V). A *c-MYC/GAPDH* ratio of 3.0 yielded 92.3% sensitivity and 81.3% specificity, considering FISH results as the standard.

Discussion

There is very little knowledge regarding the frequency and clinicopathological implications of *c-MYC* amplification in MGC. To the best of our knowledge, ours is the first study on this topic. The frequency of *c-MYC* amplification in GC, which mostly consists of non-MGCs, has been reported in various studies (10-17) and ranges from 4.0% to 38.1%. The different frequencies in the findings of previous studies may largely be due to the different methods and/or the different criteria for *c-MYC* amplification. We used two-colored FISH, and gene amplification was defined according to the presence of tight gene clusters, or when the FISH ratio was greater than 2.0. In this study, c-MYC amplification was found in 10.2% of the MGC and in 6.0% of the non-MGC samples.

MGC is often aggressive, with frequent presentation at a more advanced stage and deeper tumoral invasion than non-MGC (2, 3). There is no clear reason why MGC deeply invades the gastric wall and frequently presents at an advanced stage. Several explanations have been given for the advanced stage and the aggressive behavior of mucinous carcinoma in mucinous colon cancer studies. Perez *et al.* proposed that extracellular mucin may lead to tissue imbibition and facilitate cancer cell dispersion (19). Papadopoulos *et al.* stated that mucin interferes with the inflammatory response and the immunological recognition

Table IV. *Clinicopathological characteristics of c-MYC-amplified cases of mucinous gastric carcinoma.*

Case no.	Age (years)	Gender	Mucin (%)	Invasion depth	LN metastasis	Distant metastasis	Stage	Location of tumor
1	43	M	85	Serosa	+	–	III	Body
2	56	M	100	Serosa	+	–	III	Antrum
3	50	M	100	Serosa	+	–	III	Antrum
4	61	M	100	Serosa	+	–	IV	Antrum
5	37	F	60	Serosa	+	+	IV	Body
6	76	M	70	Serosa	+	–	IV	Antrum
7	70	M	80	Serosa	+	–	III	Antrum & body
8	58	M	75	Serosa	+	+	IV	Antrum
9	58	M	80	Serosa	+	–	III	Antrum
10	76	M	50	Serosa	+	–	III	Antrum
11	50	M	70	Muscularis propria	+	–	II	Antrum
12	74	F	100	Submucosa	–	–	I	Antrum
13	35	M	90	Serosa	+	+	IV	Antrum & body

M: Male, F: Female, LN: Lymph node.

Table V. *c-MYC/GAPDH ratio measured by real-time quantitative polymerase chain reaction in the c-MYC-amplified and non-amplified mucinous gastric carcinoma by fluorescence in situ hybridization (FISH).*

c-MYC amplification in FISH	*c-MYC/GAPDH* Mean ratio±standard deviation (range)	*p*-Value
Amplification (n=13)	23.46±34.75 (2.36-90.64)	0.048
No amplification (n=16)	2.28±1.17 (1.40-5.14)	

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

of tumor cells (20). Glasgow *et al*. suggested that the expression of drug resistance genes was responsible for the poor prognosis of patients with colonic mucinous carcinoma (21). In a stomach cancer study, Adachi *et al*. suggested that MGC arises initially as a typical adenocarcinoma and then becomes mucinous with tumor progression. This is explained by the intramural accumulation of mucin, which increases in accordance with tumor invasion through the gastric wall to an extent that is sufficient for the diagnosis of MGC (22). However, this cannot fully explain why some GCs become mucinous with tumor progression while others do not.

In our study, the correlation of *c-MYC* amplification with the depth of tumoral invasion in MGC, but not in non-MGC, may offer a possible explanation for this. Our results show that *c-MYC* amplification is highly correlated with the depth of invasion (p=0.007). In contrast, *c-MYC* amplification in non-MGC was not correlated with the depth of invasion. These results suggest that *c-MYC* amplification in MGCs may contribute to the deeply invasive property of MGC, leading to advanced tumor stages. A few studies have described the clinical implication of *c-MYC* amplification in GC, but these studies did not discriminate MGCs from non-MGCs. Mitsui *et al*. (11) and Kozma *et al*. (15) reported that there was no significant correlation between tumor stage and *c-MYC* amplification in GC (mostly consisting of non-MGC), but Kozma *et al*. reported that distant metastasis was more frequently found in c-MYC-amplified GC. Park *et al*. (10) and Buffart *et al*. (23) reported that there was no relationship between lymph node metastasis and *c-MYC* amplification in GC; these results are consistent with our results for non-MGC.

Regarding the patients' survival outcome, the mean disease-specific survival time of patients with c-MYC amplification (39 months) was shorter than the one of patients without *c-MYC* amplification (71 months) in MGC. However, the survival difference between patients with *c-MYC*-amplified MGC and those with non-amplified MGC fell short of statistical significance. In non-MGCs, *c-MYC* amplification led to a poorer disease-specific survival rate than no amplification of *c-MYC* (p=0.0343). There is a limited number of studies regarding the survival outcome of patients with *c-MYC*-amplified GC. Recently, Kim *et al*. reported that the combined gene expression of *c-MYC*, epidermal growth factor receptor (*EGFR*), and fibroblast growth factor receptor 2 (*FGFR2*) in GC was correlated with poor patient survival after cisplatin and fluorouracil chemotherapy (24). Combining their results with those of ours shows that *c-MYC* amplification may be a useful predictor of the clinical outcomes of the patients with GC, although further investigations may be needed.

Gene amplification can be detected by several methods, including FISH, Southern blotting, chromogenic *in situ* hybridization, comparative genomic hybridization, and real-time q-PCR. FISH has been generally accepted as the standard method for detection of gene amplification. However, FISH is expensive and labor-intensive. We assessed *c-MYC* status by using FISH as well as real-time q-PCR. The *c-MYC* amplification results by real-time q-PCR were well-correlated with the results obtained by FISH, with 92.3% sensitivity and 81.3% specificity. This result is generally consistent with those of previous studies that compared FISH and real-time q-PCR analyses of various types of tumors (10, 25, 26). Therefore, real-time q-PCR for *c-MYC* can be considered to be a good alternative method to FISH.

In summary, *c-MYC* amplification was found in 10.2% of 128 MGCs and 6.0% of 302 non-MGCs. *c-MYC* amplification was correlated with higher pathological stage (p=0.038) and greater invasion depth in MGC (p=0.007). In contrast, these differences in stage and invasion depth according to *c-MYC* amplification were not found in non-MGCs. Patients with *c-MYC*-amplified non-MGCs had a poorer disease-specific survival rate than those without. *c-MYC* amplification status by real-time q-PCR was highly correlated with FISH results.

In conclusion, this study shows that *c-MYC* amplification in MGC is highly correlated with advanced stage and deeply invasive MGC. The results of our study suggest that *c-MYC* amplification in MGC may be a genetic alteration that contributes to the frequent presentation of advanced stage of MGC.

Conflicts of Interest

We declare that we have no conflict of interest.

Acknowledgements

This study was supported by grant no. 04-2011-0440 from the SNUH Research Fund.

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Received May 10, 2012
Revised July 26, 2012
Accepted July 30, 2012