ANTICANCER RESEARCH *36*: 6467-6474 (2016)
doi:10.21873/anticanres.11245

Cyclins A, B, E and p27 in Endometrial Endometrioid Adenocarcinoma

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Abstract. *Background/Aim: We have previously shown that cyclin A, B and E hold prognostic significance in endometrial endometrioid adenocarcinoma. The aim of this study was to investigate the impact of cyclin-dependent kinase inhibitor p27 on cancer-specific survival and other clinicopathological variables, as well as further analyze the relationship between p27 and cyclins A, B and E and their combined relation to prognosis in the disease. Patients and Methods: The study comprised of 211 patients surgically treated for endometrial endometrioid adenocarcinoma at the Oulu University Hospital between 1992 and 2000. Tissue samples were immunohistochemically stained for cyclins A, B and E, as well as p27. Clinicopathological data were retrospectively retrieved from the patients' records. Results: In this study, universally low cyclin expression was found to be an independent, favorable prognostic factor in endometrial endometrioid adenocarcinoma. A strong correlation was found between cyclin A and cyclin B expression and weaker correlations between other cyclin and p27 pairs. Nuclear p27 expression correlated with stage and produced near-significant results in univariate survival analysis. Conclusion: Combining the expression level of different cyclins may be useful in determining the prognosis in endometrial cancer. Unfortunately, it remains unclear whether high p27 expression is a poor or a favorable prognostic factor. Further large-scale studies are required to assess the effects of cyclins and p27 in endometrial cancer.*

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Key Words: p27, cyclins, CDKs, endometrial adenocarcinoma, prognosis.

Cyclin-dependent kinases (CDKs) are a family of enzymes that play a key role in the regulation of the cell cycle. The activity of CDKs is regulated by the binding of an activator from the cyclin family and by inhibition of CDKs and cyclin-CDK complexes by cyclin-dependent kinase inhibitors (CKIs). By binding to their specific CDKs, cyclins A, B, D and E directly influence cell cycle progression. Cyclin D-CDK4/CDK6 and cyclin E-CDK2 complexes control G_1 and S phase progression. Cyclin B-CDK1 complex regulates the transition between G_2 and M phase. Cyclin A, on the other hand, has specificity for both CDK1 and CDK2 and, along with cyclins B and E, influences both S phase progression and G_2/M transition (1-2).

CKIs have been divided into two classes: the inhibitors of CDK4 (INK4) p15, p16, p18 and p19 and kinase inhibitor proteins (Kips) p21, p27 and p57. INK4s inhibit CDK4 and CDK6, whereas Kips have a more dualistic role; they inhibit CDK2-cyclin complexes but they also seem to facilitate the binding of cyclin D to CDK4/6 (3).

Understandably, the direct involvement of cyclins, CDKs and their inhibitors in the cell cycle has given rise to interest in cancer research. Deregulation of different cyclins, CDKs and their inhibitors have been shown to hold prognostic significance in neoplasms of different origin (2, 4). However, their involvement in endometrial cancer is only partially understood.

Endometrial cancer is the leading gynecological malignancy in Western countries. In Finland, the age-adjusted incidence and mortality rates in 2012 were 12.9 and 2.2 per 100,000 women, respectively (5). Endometrial cancer has traditionally been divided into type I and type II tumors. Type I tumors are typically of endometrioid subtype and thought to be associated with estrogen excess, obesity, hormone-receptor positivity and endometrial hyperplasia. Type II tumors comprise of subtypes, such as serous and clear-cell carcinoma, which develop in an atrophic endometrium (6). The most common histopathological type,

endometrioid adenocarcinoma, carries a five-year survival rate of 80% and over (7). Despite the relatively favorable prognosis, there is a need to find novel predictive and prognostic factors to ensure more aggressive treatment, where necessary.

Previously, we have analyzed the impact of cyclin A, cyclin B and cyclin E on endometrial endometrioid adenocarcinoma (8-9). In this study, we evaluate the effects of p27 expression on conventional clinicopathological and prognostic factors in the same patient population and further analyze the correlations between cyclins A, B and E, as well as p27, and their relation to prognosis.

Patients and Methods

Endometrial adenocarcinoma samples were obtained from 211 patients treated at the Department of Obstetrics and Gynecology of Oulu University Hospital, Oulu, Finland, between 1992 and 2000. The median age of the patients was 64 years (range=37-98) and median body mass index (BMI) 29.7 kg/m^2 (range=19.8-49.1). Extrafascial hysterectomy, bilateral salpingo-oophorectomy and pelvic lymphadenectomy were the operative treatments in most cases (n=206). Two patients had preoperative, 134 patients postoperative and two pre- and postoperative radiotherapy. Four patients received neoadjuvant and 45 adjuvant cisplatin-based chemotherapy.

All cases were staged according to the International Federation of Gynecology and Obstetrics (FIGO) classification 1988 and, for the purpose of this series of studies, accurately converted to FIGO classification 2009. Stage I tumors were present in 140, stage II in 30, stage III in 36 and stage IV in five patients. Histopathological examination revealed grade 1 in 112, grade 2 in 66 and grade 3 in 33 of the samples. Median follow-up time was 77 months (range=0-136). At the end of the follow-up, 53 of the 211 patients had died; 33 patients of the disease, 20 of other causes. The number of patients with all four biomarkers analyzed was 199.

Approval for the study was obtained from the Regional Ethics Committee of the Northern Ostrobothnia Hospital District.

Immunohistochemical staining. Representative tumor-containing paraffin blocks were selected and 4-μm sections were cut for immunohistochemistry. Slides were deparaffinized in xylene, followed by a declining alcohol series. Prior to adding the antibody, the samples were incubated in Tris-EDTA (Sigma-Aldrich, St. Louis, MO, USA) (pH 9.0) and boiled in a microwave oven for 2 min at 850 W and for 15 min in 350 W. Endogenous peroxidase activity was blocked by using 0.1% hydrogen peroxide in methanol solution (Envision-kit; Dako, Glostrup, Denmark).

The slides were incubated for 1 h with a mouse monoclonal p27 antibody (Leica Biosystems; Newcastle upon Tyne, UK) at a 1:200 dilution, followed by a standard avidin-biotin complex protocol using Envision staining kit (Dako). The sections were counterstained with hematoxylin and mounted with overslipping film TissueTek (Sakura, Torrance, CA, USA). Instead of antibody, phosphate-buffered saline was used as a negative control and known positive endometrial carcinoma samples from prior series were used as positive controls. p27 positivity was evaluated as the percentage of positive nuclear staining/whole tumor area in the section. Other staining patterns were evaluated separately (8-10).

Statistical analyses. All statistical analyses were carried out by using the SPSS for Mac version 21 software (International Business Machines Corp, Armonk, NY, USA). The relationships between clinicopathological variables and nuclear p27 were assessed with the Kruskal-Wallis or Mann-Whitney *U*-test. Chi-squared test was used to analyze the relationships between clinicopathological variables and cytoplasmic p27. Correlations between continuous variables were tested by Spearman's rank correlation. Receiver operator characteristic curve (ROC) was used to determine the accuracy of p27 as a discriminator between patients with a good and poor prognosis over a range of cut-off points. Cumulative survival was analyzed by Kaplan-Meier analysis. The differences between the subgroups were compared by means of a log-rank test. The Cox proportional hazards model was used in multivariate analysis to assess the independency of the prognostic factors.

Results

The median labeling index (LI) of nuclear p27 was 10% (range=0-100). Forty-seven cases had negative staining and seven were not analyzed due to a limited number of tumor sections (Figure 1).

In both grade 1 and grade 2 tumors, the median LI was 10% (range=0-100). An increase to 17.5% (range=0-100) was seen in grade 3 tumors. The differences were not statistically significant. Combined early stages (I+II) had a lower median LI of 10% (range=0-100) compared to the median LI of 17.5% (range=3-100) in advanced stages (III+IV). The difference was statistically significant (Table I).

Correlation analyses between cyclin A, cyclin B, cyclin E and p27 revealed a strong correlation between cyclin A and cyclin B (Figure 2) and weaker correlations between other cyclin pairs or p27 (Table II).

For survival analyses, a cut-off value of 7.5% for p27 was estimated from the ROC curve and the patients were grouped accordingly to low- (LI ≤7.5%) and high- (LI >7.5%) expression groups. The same method was utilized to group cyclin A, cyclin B and cyclin E expression, as described previously (8-10). In cumulative survival analysis, the patients with a low p27 LI had a five-year cancer-specific survival rate of 90%, compared to a survival rate of 81% for patients with a high LI. The difference, however, did not reach statistical significance (Figure 3A).

Cytoplasmic p27 staining was considered separately but its expression did not correlate with grade or stage. Cumulative survival analysis showed a slight tendency towards poorer survival in patients with intense cytoplasmic p27 staining but the results were not statistically significant (data not shown).

Cox proportional hazard model was used to assess the independency of different variables. A controlled analysis was run with first fitting the model with FIGO stage, grade and cyclin A, as this was estimated to best fit the data according to previously published approaches (8). In the second phase, cyclin B, cyclin E and nuclear p27 were individually added but none was able to improve the model.

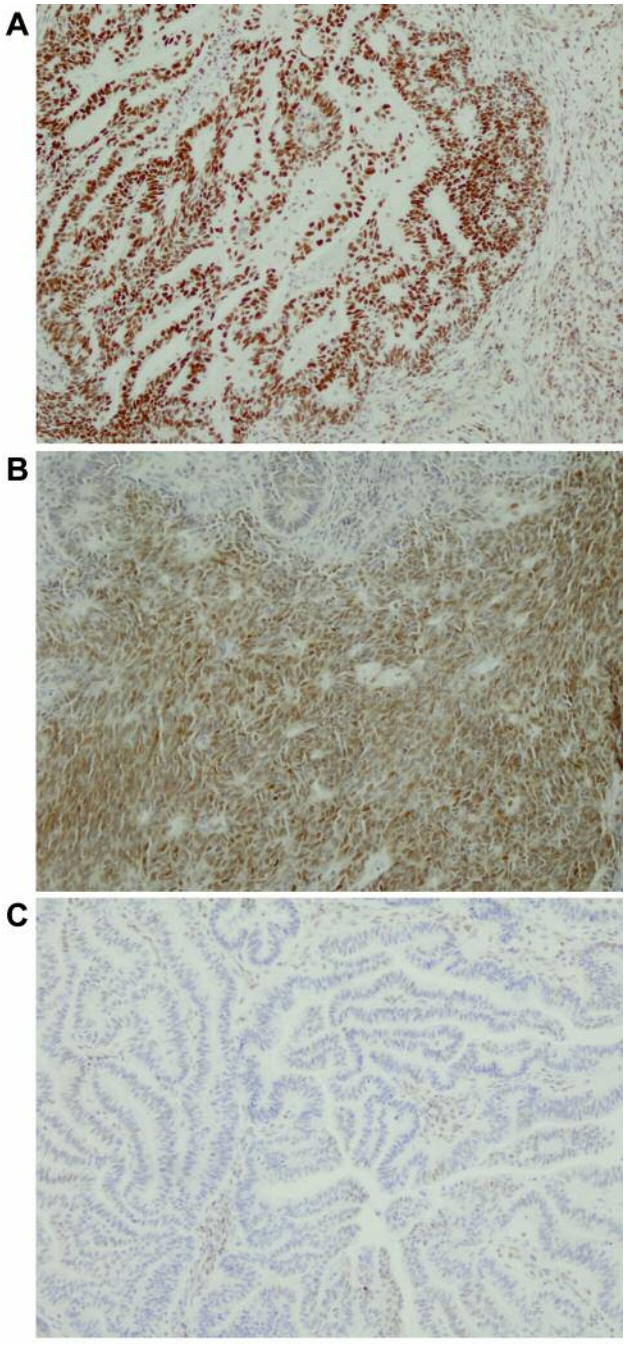

Figure 1. *p27 staining. A, Strong positive nuclear staining for p27. B, Partly cytoplasmic and nuclear staining pattern for p27. C, Negative staining for p27.*

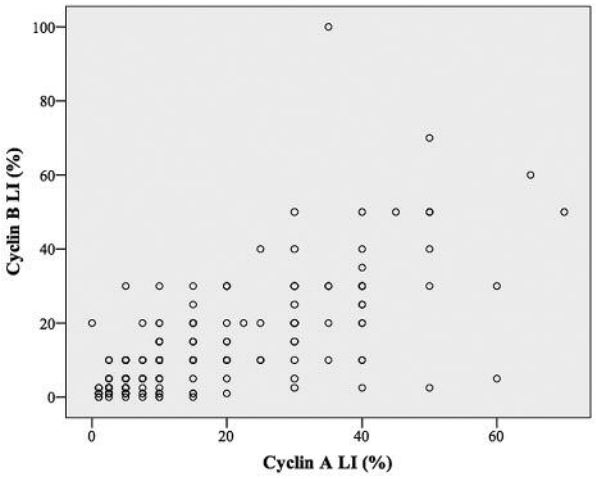

Figure 2. *Expression of cyclin A and cyclin B showing a strong correlation.*

Table I. *Labeling indexes (LI) of stage.*

Stage	Number of patients	LI median (%)	*p*-Value
I+II	164	10 (0-100)	
III+IV	40	17.5 (3-100)	0.027*

*Stage I + stage II vs. stage III + stage IV.

Table II. *Correlation coefficients between cyclin A, cyclin B and cyclin E, as well as p27.*

	Cyclin A	Cyclin B	Cyclin E
Cyclin B	0.694*		
Cyclin E	0.345*	0.305*	
p27	0.275*	0.185*	0.147**

*$p<0.01$; **$p<0.05$.

All the cyclins and p27 were analyzed as categorical variables based on the cut-off value-defined groups.

Splitting the data according to the cut-off value-defined groups revealed that cyclin A was a poor prognostic factor in univariate analysis in a subset of patients with a low cyclin B expression and *vice versa*. Based on this notion, we combined the groups in which either of the cyclins showed high expression and compared the survival to the group in which both cyclins showed low expression. Cumulative survival analysis revealed a five-year cancer-specific survival rate of 95% for patients with a low expression of both cyclins, compared to a 76% survival rate for patients with either cyclin showing high expression (Figure 3B). In the Cox proportional hazard model with stage and grade, the

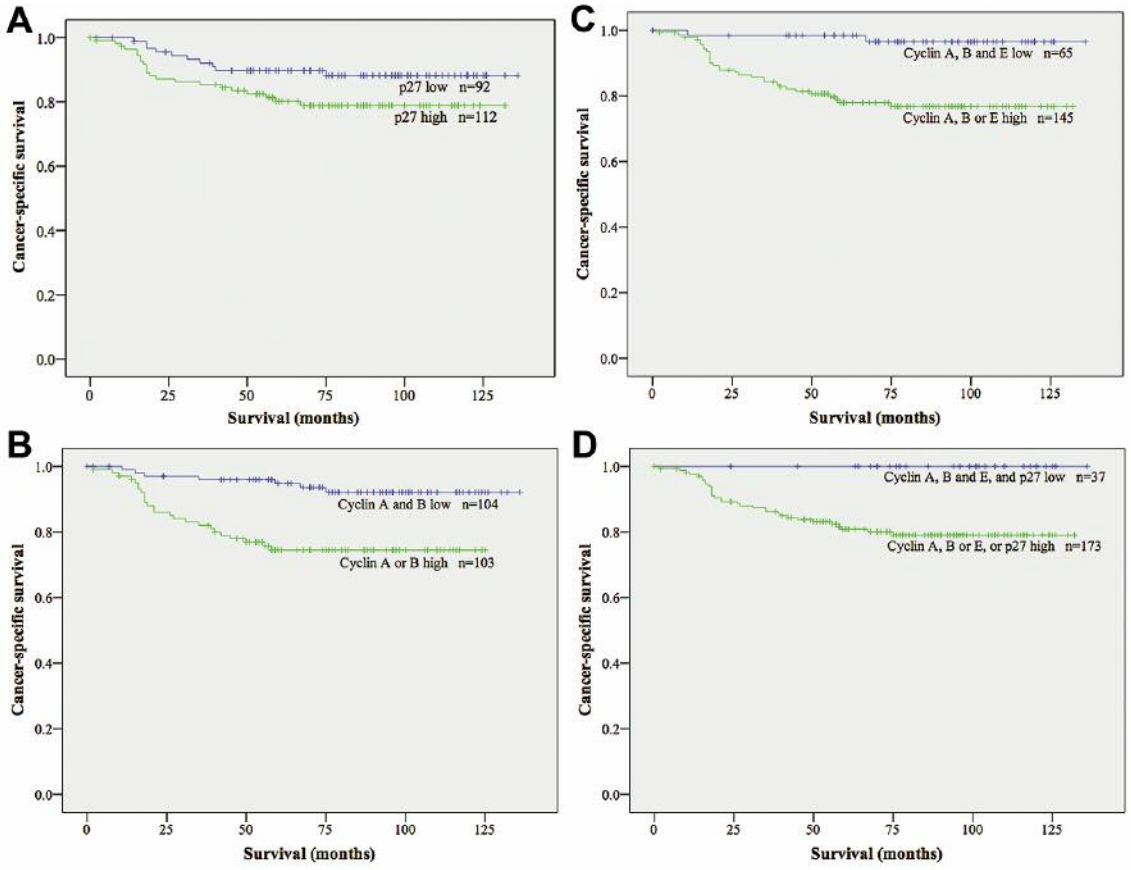

Figure 3. *Cancer-specific survival. A, Cumulative survival according to p27 as defined by the cut-off value of 7.5% (p=0.075; at 60 months p=0.069). B, Cumulative survival according to combined cyclin A and cyclin B (p<0.001; at 60 months p<0.001). C, Cumulative survival according to universally low cyclin expression (p=0.001; at 60 months p<0.001). Cyclin A, cyclin B and cyclin E cut-off value each 12.5%. D, Cancer-specific survival reached 100% when cyclin A, cyclin B, cyclin E and p27 expressions were universally low and 81% if any of the markers showed high expression (p=0.004). Crosses indicate censored cases.*

group in which either cyclin showed high expression was estimated as having a hazard ratio of 3.2 (95% confidence interval (CI)=1.3-8.0; *p*=0.013).

Similarly, there was a tendency towards poorer survival in patients with low cyclin A and B expression but high cyclin E expression. Cumulative survival analysis in the group with a universally low cyclin expression showed a five-year cancer-specific survival rate of 98%, compared to a 79% survival rate for the group in which any of the cyclins showed high expression (Figure 3C). Multivariate analysis in the Cox proportional hazard model was then performed with the following factors: high expression of any cyclin, FIGO stage and grade (Table III).

Notably, when all four biomarkers showed low expression, cancer-specific survival reached 100% (Figure 3D). However, the number of patients was not sufficient to analyze further the effects of combining all four biomarkers.

Table III. *Independent and significant prognostic factors and hazard ratios of cancer-specific death.*

Variable	Hazard ratio	*p*-Value to remove
Stage		<0.001
I	1	
II	2.6 (1.0-6.9)	
III	3.8 (1.7-8.5)	
IV	15 (4.0-55)	
Grade		0.019
1	1	
2	2.1 (0.88-5.0)	
3	3.7 (1.5-9.3)	
Cyclin expression		0.018
all ≤12.5	1	
any >12.5	5.8 (1.4-25)	

Discussion

p27 was first identified as an inhibitor of cell proliferation, more specifically a cyclin-dependent kinase inhibitor. As one might expect, low expression of p27 has been linked to poor prognosis in a number of malignant neoplasms, including gastric, lung and breast cancer (11). However, the current understanding of its effects is more dualistic in nature: p27 is an inhibitor of CDK2 but also seems to facilitate the activation of CDK4 and CDK6. Interestingly, whereas nuclear p27 staining has been shown to be beneficial for patient outcome in a number of malignancies, cytoplasmic p27 staining seems to convey a more ominous message. Based on these findings, it has been suggested that cellular localization, mediated by the phosphorylation of Thr-157, Thr-198 or Ser-10 by Akt or other enzymes, is an important part in determining the inhibitory/activating effects of p27 (3, 4).

In this study, the impact p27 had on cancer-specific survival produced only near-significant findings. Interestingly, however, a tendency towards poorer survival was observed in patients with a high nuclear p27 expression.

Likewise, previous studies have struggled to find a clear correlation between p27 expression and prognosis (12-16). Previously, Seeber *et al*. (16) showed that high p27 expression is a poor prognostic factor in multivariate analysis but only in a subset of patients with perinecrotic hypoxia-inducible factor 1α (HIF-1α) expression. Furthermore, Dellas *et al*. (13) showed that combined loss of p27 and phosphatase and tensin homologue (PTEN) carried a more favorable prognosis in multivariate analysis. Interestingly, however, in two large-scale studies by Steinbakk *et al*. (17-18), a correlation between low p27 expression and poor prognosis was found in univariate analysis in the curettages of early stage (FIGO I and I-II) tumors. Overall, survival analyses have produced mixed results and it remains unclear whether nuclear p27 expression should be regarded as a poor or favorable prognostic factor in endometrial cancer.

Contrary to the vast majority of studies, we also separately considered cytoplasmic p27 expression but did not find an association with survival or other variables. In accordance with our findings, Nycum *et al*. (12) found no correlation between cytoplasmic p27 staining and survival or other clinicopathological variables. Mutations of the gene encoding p27 are thought to be extremely rare in malignant tumors, whereas other mechanisms, particularly cytoplasmic mislocalisation, have been suggested to account for the role of p27 in tumorigenesis (4). Our findings do not support this mechanism in endometrial cancer.

We previously reported that cyclin A is an independent prognostic factor in endometrial endometrioid adenocarcinoma (8), whereas cyclins B (9) and E (10) are prognostic factors only in univariate analysis. In this study, we further analyzed the relationship between the cyclins and found that a universally low expression is associated with a more favorable cancer-specific survival in multivariate analysis.

We also found a strong correlation between cyclin A and cyclin B and weak to moderate correlations between other cyclin pairs or p27. Some correlations between cyclins have previously been analyzed in endometrial cancer (19-21); however, no studies have reported a strong correlation between cyclin A and cyclin B, although reports on other malignant neoplasms have found a strong correlation between them (22-24). These findings may be attributable to the fact that both cyclin A and cyclin B are expressed in the cell cycle roughly at the same time, share transcription promoters and proteolysis machinery and may, thus, to some extent, reflect similar changes in cell proliferation (25). In this sense, combining cyclin A and cyclin B in survival analyses may be useful, as shown in this study.

In the present series, we found a correlation between nuclear p27 expression and advanced stage, but not with grade. Previously, Nycum *et al*. (12) reported a correlation between combined nuclear and cytoplasmic p27 expression and stage. However, there is only one study by Watanabe *et al*. (26) reporting a correlation between nuclear p27 expression and stage. Along with others, they also found a correlation between p27 and grade (13, 16, 27). On the other hand, divergent findings also exist (14-15, 28). The divergence of findings may be explicable in terms of various immunohistochemical stainings, interpretation techniques and data grouping methods used.

The patients in this study underwent surgery during a time when pelvic lymphadenectomy was routinely performed, which may have had a positive effect on the accuracy of staging. All patients were treated in the same facility, Oulu University Hospital, and all samples treated with a uniform protocol. Immunohistochemical stainings were analyzed by two investigators blinded to the clinical data. Systematic surgical staging was performed in accordance with the FIGO (2009) criteria. Follow-up was organized systematically and was sufficiently long to show deaths from the disease.

The disadvantages of this work include the retrospective study setting and the subjectivity of evaluating immunohistochemical staining. Furthermore, a small number of patients received chemo- and/or radiotherapy for their tumors prior to surgery, possibly affecting the p27 and cyclin levels in some samples. Many patients also received postoperative therapy, as was the standard treatment at the time. The benefit of such treatment has since been re-evaluated and it is currently not recommended for low-risk patients (29). The effects of the treatment on patient survival have likely been minimal, thus not affecting the integrity of the study.

In conclusion, we have shown here that universally low cyclin expression is an independent, favorable prognostic factor in endometrial endometrioid adenocarcinoma.

Furthermore, we found a correlation between nuclear p27 expression and advanced stage. Nuclear p27 expression also produced near-significant results in univariate survival analysis. Analyses on the effects of p27 in endometrial cancer have produced mixed results in previous studies, thus remaining unclear whether high p27 expression is a poor or a favorable prognostic factor. Further large-scale studies are required to assess the effects of cyclins and p27 in endometrial cancer.

Acknowledgements

The Authors thank Mr. Risto Bloigu, MSc, for his assistance in the execution and evaluation of the survival analyses. They also thank laboratory technicians Mrs. Erja Tomperi and Mrs. Mirja Vahera.

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Received September 12, 2016
Revised September 25, 2016
Accepted September 26, 2016