CYCLIN D1 GENE G870A POLYMORPHISM PREDICTS RESPONSE TO NEOADJUVANT RADIOTHERAPY AND PROGNOSIS IN RECTAL CANCER

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**Conflict of Interest Notification**

We certify that there are no personal or financial relationships or other interests with regard to this manuscript that might be construed as constituting a conflict of interest for any author.
**Purpose:** The CCND1 gene encodes the cyclin D1 protein, which plays an important role in carcinogenesis. Increased oncogenic potential can be obtained through exon 5 mutations or alternate splicing modulated by a common G870A polymorphism, both of which are involved in nuclear export defect. The aim of this study was to investigate those genetic variations and their prognostic significance in rectal cancer.

**Methods and Materials:** Seventy rectal cancer patients treated by neoadjuvant radiotherapy were included in the study. CCND1 exon 5 mutations were screened and the G870A polymorphism was assessed for correlation with clinical variables, tumor response, and patient outcome.

**Results:** No exon 5 mutation was found. Concerning the G870A polymorphism, the A/A variant was significantly associated with radiosensitivity ($p = 0.022$). Moreover, patients harboring the A-allele were correlated with a lower risk of local failure ($p = 0.017$). Also, combination of the G870A polymorphism with the post-therapeutic lymph node status allowed the elaboration of a prognostic index, which accurately distinguished subgroups of patients with predictable recurrence-free ($p = 0.003$) and overall ($p = 0.044$) survival.

**Conclusions:** While CCND1 exon 5 mutations are rare in rectal cancer, G870A polymorphism is a frequent variation that may predict radiosensitivity and prognosis.

Cyclin D1, Single nucleotide polymorphism, Radiotherapy, Predictive marker, Rectal cancer.
INTRODUCTION

During the last decade, evidence supporting the use and benefits of neo-adjuvant radiotherapy (RT) for locally advanced rectal cancers has accumulated. Particularly, it has been shown that preoperative irradiation improves local control (1–3) and survival (1, 2, 4). In addition, RT can induce tumor regression, which may allow subsequent radical surgery in a primarily non-resectable tumor (5), and increases the probability of sphincter-saving procedures in low tumors (6, 7). Moreover, 5-year survival studies identify complete pathologic response, defined as the absence of any residual viable tumor cells in the surgical specimen, as an important prognostic factor (8).

However, tumor response to irradiation varies widely among individuals, and in rectal cancer, RT can be ineffective in up to 50% of the patients. The selection of patients for neoadjuvant RT is currently based on clinical parameters, rectal endosonography, and CT-scan finding (9), but these parameters are unable to predict response. Identification of predictive markers of cancer response to preoperative RT is of clinical importance to enable tailor-made individualization of therapy. One common approach to find key molecular markers is to study polymorphisms (10, 11) or mutations (12) of cancer-related genes.

The CCND1 gene encodes the cyclin D1, a protein that plays an important role in promoting cellular proliferation (13) and regulating transcription (13, 14). Cyclin D1 is overexpressed in many types of cancers (15). This overexpression is tumor-type specific and can result from chromosomal translocation (16), gene amplification (17, 18) or induction by oncogenic signals such as Ras (19). However, it appears that cyclin D1 overexpression by itself may not be sufficient to drive malignant transformation (20, 21). Instead, one important oncogenic factor could be a defect in cyclin D1 nuclear export (22–25). The localization of cyclin D1 depends on its phosphorylation of Thr-286 by glycogen synthase kinase-3beta
(GSK-3β), which marks cyclin D1 for nuclear export by the CRM1 exportin. Sequences regulating this process are located on exon 5, and expression of a constitutively nuclear cyclin D1 can be obtained through two distinct mechanisms. On the one hand, exon 5 mutations can block Thr-286 phosphorylation and/or CRM1 binding (22, 26, 27). Such mutations have recently been described in a few cases of endometrial (26) and esophageal (27) carcinomas. On the other hand, CCND1 D1 may undergo alternative splicing leading to the expression of the cyclin D1b isoform, where exon 5 is replaced by a truncated portion of intron 4 (24, 25, 28).

It has been suggested that the splicing of the CCND1 transcript is modulated by a common single nucleotide polymorphism (SNP) located at the exon 4/intron 4 boundary, at nucleotide 870 (codon 241) (28). The G-allele codes for an optimal splicing site, inducing the production of the D1a transcript, and the A-allele has been predicted to constrain intron 4 excision, thus resulting in the truncated D1b transcript (26). Although a significant number of studies have demonstrated the implication of the CCND1 G870A polymorphism in cancer susceptibility [reviewed in (29)], the potential role of this SNP in the management of cancer therapy remains to be addressed.

The aim of this study was to investigate whether the above-mentioned CCND1 genetic variations, associated with a constitutive nuclear protein, may influence either the pathologic response to preoperative radiotherapy or the prognosis in a series of rectal cancer patients.

METHODS AND MATERIALS

Patients

A total of 70 Caucasian patients with rectal adenocarcinoma were enrolled in this study from 1996 until 2001. They consisted of 44 men and 26 women, with a median age of 64
years at the time of diagnosis (range, 39–81 years). Before initiation of therapy, they were staged using the 1997 TNM classification based on a clinical examination, endoscopic and endorectal ultrasonography (ERUS) evaluation, and computed tomography of the thorax and abdomen. Patients were treated homogeneously at the Val d’Aurelle Paul-Lamarque Cancer Center in Montpellier by preoperative radiotherapy followed by surgical resection. A detailed description of the patients’ clinicopathological characteristics is presented in Table 1. For all patients, informed consent for the use of clinical records and tissues for research purposes was obtained.

*Preoperative radiotherapy*

Patients were treated in the supine position with a three-field (posterior and opposed laterals) isocentric technique using 18-MV photon beams daily, five times a week. The daily dose at the isocenter was 1.8 Gy; the total dose to the entire pelvis was 45 Gy. For a sphincter preservation approach, twenty-nine patients with a very low rectal cancer, uT2 or uT3 on ERUS initial staging and M0 by CT scan, received a boost dose of 15 Gy. The boost volume covered the primary tumor plus a 1.5-cm margin using a three-field (posterior and oblique) technique.

*Surgical and pathological modalities*

The median time between the first day of radiotherapy and surgery was 10 ± 3.9 weeks. The choice of the surgical procedure was at the surgeon’s discretion. A total proctectomy with complete excision of the mesorectum was systematically performed. For upper tumors of the lower third of the rectum, the rectal section was done during the abdominal approach, close to the levator ani muscle, and then a per anum mucosectomy was made. For lower tumors, the distal pole of the specimen was dissected through the anus, immediately above the dentate
line. For tumors located at or near the superior end of the sphincter ring, an inter-sphincteric resection was performed with partial or complete removal of the internal sphincter. Bowel continuity was restored by coloanal anastomosis, preferably with a J colonic pouch or directly for a narrow pelvis in obese males. Abdomino-perineal resection was performed when the patients had a compromised sphincter function, when the tumor invaded the external anal sphincter or the levator ani muscle or in case of a bulky tumor within a narrow pelvis. The operative specimen was staged according to the 1987 UICC pTNM staging system. All patients had a R0 resection with lateral margins > 1 mm.

Postoperative treatment

Postoperative management of enrolled patients was performed according to French standards in use during the inclusion time. In particular, patients with a post-therapeutic positive nodal status received an adjuvant regimen of fluorouracil and leucovorin. Moreover, patients who developed local or distant recurrence were treated by various chemotherapy regimens or a second surgical resection.

Assessment of radiotherapy effects

Evaluation of the pathologic response to preoperative radiotherapy was based on the tumor regression grade (TRG), which was assessed on the resected surgical rectal carcinoma. The entire primary tumor was paraffin-embedded and regression was semiquantitatively determined by histopathologic examination of residual carcinoma cells versus fibrosis or mucin pools, as described by Dworak et al. (30).

The TRG ranges from TRG 0 when no fibrosis is visible (no regression), to TRG 4 when no viable tumor cells are detected (complete response). TRG 1 = dominant tumor mass and obvious fibrosis or mucin; TRG 2 = dominantly fibrotic or mucinous changes, with few tumor
cells or groups; TRG 3 = very few tumor cells in fibrotic or mucinous tissue. The same trained pathologist, blinded to patients’ characteristics, classified all tumors. Patients with TRG 0 or 1 were defined as nonresponders, whereas those with TRG 2, 3 or 4 were classified as responders.

Follow-up

All patients were seen on regular follow-up including clinical history, physical examination, laboratory investigations, abdominal ultrasonography, chest X ray, and endoscopy. They were followed postoperatively semi-annually until death or the closing date of the study. Any regrowth of the tumor within the pelvis was considered as a local recurrence. The diagnosis of a pelvic recurrence was preferably proven by histology and/or cytology. However, in the majority of cases, the diagnosis was made on clinical and radiological grounds. Data collected were entered prospectively into the registry database. The median follow-up was 6.5 years (range, 0.6–9.5 years).

DNA extraction

For each patient, a blood sample was collected on the day of clinical diagnosis, and four pretherapeutic endoscopic biopsies from the macroscopic tumor area were performed. The biopsies were frozen immediately after resection in liquid nitrogen. A 5-mm-thick section was cut to estimate the percentage of tumor cells, using haematoxylin and eosin staining. The remaining biological material was used for isolation of tumor DNA with the TRIZOL® Reagent (Invitrogen, Cergy Pontoise, France), according to the manufacturer’s recommendations. Non-tumor DNA was extracted from the blood samples with QIAamp DNA Blood Maxi Kit (Qiagen, Courtaboeuf, France), according to the manufacturer’s instructions.
Mutation analysis

To search CCND1 mutations, two primers were designed to amplify a sequence encompassing the exon 5 coding sequence of the CCND1 gene (GenBank Accession number AF511593). DNA extracted from rectal tumor biopsies was used as a template for PCR amplification. The 25-µL reaction mix contained 1× PCR buffer (Qiagen), 200 µmol/L dNTP, 0.4 µmol/L of each forward (5'-GTGAAGGCCTGGTTGGAC-3’) and reverse 5’-GAATGAAGCTTTCTCTCTTG-3’) primer, 2.5 units of HotStarTaq DNA Polymerase (Qiagen), and 100 ng of genomic DNA. PCR was carried out in a TGRADIENT Thermocycler (Whatman-Biometra, Goettingen, Germany) with an initial denaturation at 95°C for 15 minutes to activate the enzyme, followed by 35 amplification cycles consisting of a denaturation step at 94°C for 30 seconds, a primer annealing step at 60°C for 30 seconds, and an elongation step at 72°C for 40 seconds. A final extension step at 72°C for 10 minutes completed the reaction. The PCR products were verified on agarose gel and subsequently purified by an enzymatic digestion with exonuclease I (Amersham Biosciences, Orsay, France) and shrimp alkaline phosphatase, (Roche Applied Sciences, Meylan, France), according to the manufacturer’s instructions. Direct sequencing of the purified amplicons was done with a 3130 Genetic Analyzer (Applied Biosystems, Courtaboeuf, France) using the Bigdye® terminators v1.1 cycle sequencing kit (Applied Biosystems).

SNP genotyping

The determination of the CCND1 G870A polymorphism was achieved through PCR-RFLP as previously described (31) with some modifications. Briefly, a fragment of 167 bp containing the polymorphic site of interest was amplified by PCR from blood-extracted DNA. Amplification was performed in a 50-µL volume containing 1× PCR buffer (Invitrogen), 1.5
mmol/L MgCl₂, 200 µmol/L dNTP, 0.4 µmol/L of each forward (5’-GTGAAGTTCATTTCCAATCCGC-3’) and reverse (5’-GGGACATCACCCTCATTAC-3’) primer, 2.5 units of Taq polymerase (Invitrogen), and 150 ng of genomic DNA. The reaction was initiated by a denaturation step at 94°C for 1 minute, followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds with a final extension at 72°C for 10 minutes. The PCR products were digested by the NciI restriction enzyme (Promega, Charbonnieres, France) following the manufacturer’s guidelines, and the DNA fragments were visualized on a 2% NuSieve/1% agarose gel stained with ethidium bromide (Fig. 1). The A/A variant was identified by a non-cleaved 167-bp product. The G/G variant produced two fragments of 145 and 22 bp, and three fragments of 167, 145, and 22 bp were obtained for the heterozygous A/G variant.

**Statistical considerations**

The primary focus of this study was the pathologic response to neoadjuvant treatment. The second point of interest was the patient outcome with the development of local recurrence and overall survival. Categorical variables were reported by means of contingency tables. Furthermore, for continuous variables, median and range were computed. To investigate their associations with the clinical, pathological, and biological parameters, univariate statistical analyses were performed for categorical variables using Pearson’s χ² test or Fisher’s exact test when applicable. The relationship between the CCND1 G870A polymorphism and each patient’s clinicopathological characteristic was also studied. Moreover, multivariate analyses were carried out using logistic or Cox regression with stepwise procedure. Odds and hazard ratio with 95% confidence intervals are presented. All reported P-values are two-sided, and the significance level was set at 5% (P < 0.05). All survival times were calculated from the date of diagnosis. Locoregional-free and overall survival rates were estimated according to the
Kaplan-Meier method using the closing date of the study (September, 2006) as the endpoint measure. All statistical analyses were done with the STATA 9.0 software.

RESULTS

**CCND1 exon 5 mutation analysis**

For all patients, histopathological examination of slides from pretreatment tumor biopsies was performed. Mutation analyses were carried out on DNA extracted from biopsies displaying more than 30% of tumor cells on the corresponding haematoxylin- and eosin-stained slides. Direct sequencing results of CCND1 exon 5 were available for 51 patients. No mutation was found in this series.

**CCND1 G870A SNP genotyping**

Genomic DNA extracted from blood samples was available for all 70 patients enrolled in this study. Amplification of the 167 bp fragment, encompassing the polymorphic site of interest located at codon 241, was successful for all isolated DNA. Analysis of the SNP showed that 15.7% (11 of 70) of the patients were homozygous for the A-allele (A/A genotype), 27.1% (19 of 70) were homozygous for the G-allele (G/G genotype), and 57.2% (40 of 70) were heterozygous (A/G genotype).

**Correlation between CCND1 G870A polymorphism and response to radiotherapy**

Among the 70 patients included in this study, assessment of the response to therapy was only possible for 65 patients. Thirty (46.2%) cases were non responders, with 3 patients displaying no regression at all (TRG0) and 27 presenting a poor tumor regression (TRG1).
Thirty-five (53.8%) patients were good responders with 16, 14, and 5 patients evaluated as TRG2, TRG3, and TRG4, respectively. Associations between the pathologic response to preoperative radiotherapy and patient characteristics were analyzed by univariate analysis. No statistical significance was found between the pathologic response and classical clinicopathological parameters such as gender, age, tumor grade, presence of synchronous metastases, or pretherapeutic TNM stage. Concerning total irradiation doses, 50% (19 of 38) of patients that received 45 Gy treatment were good responders, compared with 59.2% (16 of 27) for 60 Gy irradiation. Nevertheless, this slight increase of good responders in the 60 Gy irradiated patient group was not statistically significant ($p = 0.461$). In contrast, patients harboring the A/A variant for the CCND1 G870A polymorphism were significantly associated with a good tumor regression ($p = 0.022$; Table 2). Indeed, 90% (9 of 10) of TRG-evaluable patients harboring the A/A genotype were good responders, compared with 42.5% (17 of 40) and 60% (9 of 15) for patients having the A/G and G/G genotypes, respectively. As with the univariate analysis, a multivariate analysis by stepwise logistic regression showed that the G870A genotype was the only factor associated with the neoadjuvant therapy effects. The presence of the A-allele was strongly correlated with the pathologic response (odds ratio 10.0, 95% confidence interval, 1.2–84.7; $p = 0.034$).

**Correlation between CCND1 G870A polymorphism and local recurrence**

Among the 70 patients included in this study, 8 experienced local recurrence. The relationship of patient characteristics with local failure was evaluated for all study participants using univariate analyses. No correlation was found between local recurrence and gender, tumor grade, the pretherapeutic TNM stage, radiotherapy regimen, response to radiotherapy, the pT or the post-treatment lymph node status (pN) stages. Although no significant association was found, none (0/11) of the patients harboring the A/A genotype developed a
local recurrence throughout the follow-up period of the study. However, when considering the A/A and A/G genotypes together, those patients presenting the A-allele were significantly associated with a lower risk of local recurrence compared with patients having the G/G genotype ($p = 0.017$). Indeed, 5.9% (3 of 51) of patients harboring the A-allele developed a local recurrence, compared with 26.3% (5 of 19) for patients having the G/G genotype. A multivariate analysis by stepwise Cox regression demonstrated that not only the G/G genotype ($p = 0.020$) but also that the positive node (pN+) stages ($p = 0.011$) were associated with an increased risk of developing a local recurrence (Table 3). Based on these data, a numerical score was derived for those two variables, whose regression coefficients for the Cox proportional hazard analysis were similar. A value of 0 was attributed to A/A or A/G genotypes and node-negative (pN-) stages, while 1 point was given to G/G genotype and pN+ status. A prognostic index (PI) was calculated by adding the individual scores for the G870A genotype and pN status. This prognostic index was found to be strongly associated with time to local recurrence ($p = 0.003$; Fig. 2) and distinguished three subgroups of patients. Patients presenting the A-allele and a pN- status formed the low risk group (PI = 0), whereas patients with the G/G genotype and a pN+ status formed the high risk group (PI = 2). The intermediate risk group comprises patients with A-allele and a pN status, and patients with the G/G genotype and a pN- status (PI = 1).

**Correlation between CCND1 G870A polymorphism and survival**

Survival analyses were performed for the 58 individuals that did not have synchronous metastases. Thirty-nine patients were alive at the end of the study with a survival rate of 98.3% at one year and 77.5% at five years. Among all the factors individually studied, particularly the clinicopathological parameters, the response to therapy and the G870A polymorphism, no relationship was found with overall survival. However, considering the
G870A polymorphism and the pN status together, a significant correlation was found between the resulting prognostic index and overall survival ($p = 0.044$).

**DISCUSSION**

The identification of molecular markers that predict preoperative radiotherapy effectiveness and/or patient outcome is of major importance in the management of rectal cancer. Such markers are powerful tools that would help to identify the patients who could benefit from treatment. On the other hand, prediction of resistance to irradiation would help to select candidates for more intensive treatment with concomitant chemotherapy, or alternative strategies such as targeted therapies (32). The aim of our study was to assess the potential predictive role of genetic alterations of the cyclin D1 cancer related gene.

Given its implication in cell cycle control (13) and gene transcription (13, 14), it is not surprising that cyclin D1 plays an important role in tumorigenesis. Cyclin D1 is overexpressed in many types of malignancies (15). In particular, protein accumulation has been described in 40% of squamous carcinomas of the head and neck, 20% of prostate cancers, and 60% of breast cancers. In colorectal cancer, this overexpression is observed in 40% of patients and is suggested to be an early event during carcinogenesis (33). In this localization, clinical studies have suggested that cyclin D1 overexpression may predict disease recurrence (18, 34), whereas no data are available regarding response to treatment. Nevertheless, it has been shown that cyclin D1 overexpression could be a determinant of chemotherapy and radiation effectiveness in head and neck (35, 36), breast (37, 38), and oral (39) cancers.

Besides expression alterations, cyclin D1 genetic variations involved in dysregulation of the protein localization have been reported. A defect in cyclin D1 nuclear export increases its
oncogenic potential, thereby rendering it able to promote malignant transformation independently of cooperating oncogenes (22–25), contrary to overexpression by itself. To determine whether cyclin D1 is subject to mutations that inhibit its nuclear export in rectal cancer, we sequenced the CCND1 exon 5 in our set of primary rectal carcinoma. Indeed, this region contains the sequences regulating the protein localization. Our data show that CCND1 exon 5 mutations, if they exist, are probably a rare event in rectal cancer. Recently published works reported such alterations in endometrial (26) and esophageal carcinoma (27). However, the mutation rate (around 3%) was very low in both studies. Taken together, those results underscore just how low the frequency of those tumor-derived genetic alterations is.

In contrast, the G870A SNP is a fairly common genetic variation associated with cyclin D1 nuclear export. In our homogeneous series of Caucasian patients, the calculated allele frequency distributions were 44.3% for the A-allele and 55.7% for the G-allele, which is comparable with the mean prevalence described in the NCBI SNP database for this ethnicity (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=603965). This polymorphism does not modify the amino acid sequence. However, since it is located at the exon 4/intron 4 boundary, this SNP is supposed to influence the frequency of alternate splicing. As predicted, it has been shown (28, 40) that the A-allele involves preferentially an absence of intron 4 excision. The resulting D1b transcript leads to the expression of a truncated protein with a completely divergent C-terminal domain encoded by a part of intron 4, where the nuclear export regulatory sequences are missing.

In our series, the A/A genotype was significantly associated with a good response to neoadjuvant radiotherapy, both in univariate ($p = 0.013$) and multivariate analyses ($p = 0.034$). Determination of CCND1 G870A polymorphism could provide useful information to select patients who respond to common neoadjuvant radiotherapy. Moreover, cyclin D1 is a crucial target for various types of human cancers, and antisense strategies have been
performed to suppress the malignant potential of carcinomas. Notably, this strategy has been reported to enhance chemosensitivity in head and neck cancer cells (41), induce apoptosis in esophageal squamous carcinoma (42) and inhibit angiogenesis in colon and gastric cancer (43). Based on these data, it can be considered that the G870A polymorphism, associated with cyclin D1 localization, may be a useful predictive marker of tumor response for cyclin D1 targeted therapies.

Local recurrence is a critical problem in rectal cancer. Actually, it is difficult to cure, and is associated with a poor prognosis. It also has a profound negative effect on quality of life, as it involves severe suffering for the patient, with pain, bleeding, ulceration, and fistulation as common associated symptoms. Our data suggest that local control is significantly correlated with the G870A SNP, the A-allele being associated with lower local recurrence rates ($p = 0.017$). By combining this SNP with the post-therapeutic lymph node status, a prognostic index was created, allowing classification of patients into three groups, whose risk of local recurrence and survival can be more accurately predicted. In particular, patients with the G-allele and a pN+ status were associated with a less favorable outcome. Implication of the pN(+) status in development of local recurrence in rectal cancer patients treated by chemoradiotherapy has been shown recently (44). Moreover, in another recent retrospective study (45) including 90 rectal cancer patients, 21 polymorphisms in 18 genes linked to cancer progression were examined. Using classification and regression tree analysis, the authors pointed out five variables associated with risk of local recurrence: once more, the pN status, but also SNPs in interleukin 8, intracellular adhesion molecule-1, fibroblast growth factor receptor 4, and transforming growth factor-beta genes. However, no correlation was found risk of local recurrence and CCND1 G870A polymorphism. This discrepancy with our data can be explained by the fact that patients enrolled in that study were treated in two different centers by two different modalities, i.e., neoadjuvant or adjuvant radio- and chemotherapy.
Implication of G870A polymorphism in prognosis has also been addressed by Zhang et al. (46) in different clinical settings. In their series, 39 metastatic colorectal cancers were treated with the monoclonal anti-EGFR antibody cetuximab. The G-allele was associated with improved survival: patients with the G/G and A/G genotype survived for a median of 8.7 months compared with 2.3 months for the A/A genotype. It has been shown that the A-allele –mainly the A/A genotype– is associated with the expression of cyclin D1b (28, 40), and with increased cancer risk or early onset in colorectal, gastro-intestinal, head and neck, esophageal, and other cancer types (29). Besides its role in regulating cell cycle progression, cyclin D1 can enhance or repress transcription by forming physical associations with several transcription factors or transcriptional coregulators (13, 14). The truncated isoform lacks, however, the LxxLL motif, which is important for coactivator recruitment (47, 48). Combined with the fact that the protein remains nuclear, the transcriptional activity of cyclin D1b may be significantly altered (29). Based on these data, one could hypothesis that cyclin D1b-driven carcinogenesis, associated with the A-allele, may involve an array of specific mechanisms and pathways that lead to more radiosensitive and less aggressive tumor cells.

The results presented in this study are based on a moderate number of patients. Despite this limitation, our patients’ population was treated homogeneously by neoadjuvant radiotherapy at the same cancer institute, and data obtained raise interesting clinical implications.

In conclusion, CCND1 G870A polymorphism is a potential new prognostic marker in rectal cancer. Our study demonstrated that this SNP was a strong predictor of response to radiotherapy and risk of local recurrence, both in univariate and multivariate analyses. An association with overall survival was also found when combining the polymorphism with the post-therapeutic lymph node status. Further clinical trials are needed to confirm the relevance of our results.
REFERENCES


FIGURE LEGENDS

Fig. 1. Detection of the CCND1 G870A polymorphism by PCR-RFLP.
M, 100-bp ladder; lane 1, non-digested product; lane 2, G/G homozygous genotype (145 bp); lane 3, A/A homozygous genotype (167 bp); lane 4, A/G heterozygous genotype (167 and 145 bp).

Fig. 2. Kaplan-Meier curves for locoregional recurrence-free survival in rectal cancer according to the prognostic index (PI).