EUROPEAN JOURNAL OF CANCER 46 (2010) 2304–2313

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The loss of the CBX7 gene expression represents an adverse prognostic marker for survival of colon carcinoma patients

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ARTICLE INFO

Article history:
Received 27 January 2010
Received in revised form 28 April 2010
Accepted 6 May 2010
Available online 9 June 2010

Keywords:
CBX7
Colorectal adenocarcinomas
Immunohistochemistry
FISH

ABSTRACT

We have previously shown that CBX7 expression is associated with a more malignant phenotype in thyroid cancer. On this basis, we decided to investigate its possible prognostic value in colorectal cancer (CRC). CBX7 expression has been analysed by immunohistochemistry in tissue microarray (TMA) specimens obtained from a large series of sporadic CRC resections ($n = 1420$). The CBX7 expression data have been correlated with several clinico-pathological parameters. CBX7 expression is reduced or absent in a significant number of CRC samples in comparison to the normal colonic mucosa and the loss of CBX7 expression correlates with a poor outcome of CRC ($p < 0.001$). The block of CBX7 expression seems to occur at a transcriptional level since quantitative RT-PCR analysis showed a reduced CBX7-specific mRNA levels in CRC samples versus normal counterpart tissue (up to more than 50-fold). Finally, the restoration of CBX7 expression in two CRC cell lines reduces their proliferation rate suggesting a role of the loss of CBX7 expression in the progression step of colon carcinogenesis.

Therefore, the data reported here indicate that the evaluation of CBX7 expression may represent a valid tool in the prognosis of colon cancer since a reduced survival of CRC patients is associated with the loss of CBX7 expression.

1. Introduction

Colorectal carcinoma (CRC) is the third most common cancer in the USA,[1] with survival estimates at 5 years of 64%.[1] The molecular events underlying the genesis of CRC, both familiar and sporadic, have been well determined.[2–4] Approximately 85% of CRCs arise through the so-called chromosomal instability (CIN) pathway, characterised by microsatellite stability

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Abbreviations: CRC, colorectal carcinoma; G6PD, glucose-6-phosphate dehydrogenase; FISH, fluorescence *in situ* hybridisation; qRT-PCR, quantitative RT-PCR; MMR, mismatch-repair.

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doi:10.1016/j.ejca.2010.05.011

(MSS).[5] The remaining tumours are characterised by microsatellite instability (MSI) due to inactivation of mismatch repair (MMR) genes.[5] There are, however, several unknown events that regulate the invasiveness of CRC and might influence the survival of the patients.[6] The knowledge of these events may help to identify subgroups with more aggressive disease, thereby, leading to more individualised treatment protocols.[7]

CBX7 is a chromobox family protein and a member of the Polycomb repressive complex 1 (PRC1), which together with the PRC2, maintains developmental regulatory genes in a silenced state.[8–10] Mouse Cbx7 associates with facultative heterochromatin and inactive X chromosome, suggesting a role of the Cbx7 protein in the repression of gene transcription.[11,12] Our group has previously reported that CBX7 gene is drastically downregulated in thyroid carcinomas and its expression progressively decreases with malignant grade and neoplastic stage.[13] Indeed, CBX7 protein levels decreased in an increasing percentage of cases going from benign adenomas to papillary, follicular and anaplastic thyroid carcinomas. Very recently the loss of CBX7 expression has been shown to be related to progression of urothelial carcinoma of the bladder.[14] These data suggest that the loss of CBX7 expression may be strictly correlated with the acquisition of invasiveness accompanied by the loss of the epithelial features and the gain of a mesenchymal phenotype, a process known as epithelial-mesenchymal transition (EMT). Consistent with this hypothesis, we have recently demonstrated that the CBX7 is able to positively regulate E-cadherin expression that plays a critical role in maintaining normal epithelial cell morphology, by interacting with histone deacetylase 2 and inhibiting its activity on the E-cadherin promoter thereby accounting for the correlation between the loss of CBX7 expression and a highly malignant phenotype.[15]

Therefore, CBX7 could be involved in cancerogenesis and may be a candidate to predict the prognosis also of other cancer types. Hence, the aim of this study was to investigate a possible prognostic role of CBX7 detection in CRC.

2. Materials and methods

2.1. Human colorectal tissue samples

Human neoplastic colorectal tissues and normal mucosa tissues were obtained from surgical specimens and immediately frozen in liquid nitrogen at the Department of Pathology, University of Basel, Switzerland. The tumour samples were stored at –70 °C.

2.2. Tissue microarray (TMA) and immunohistochemistry (IHC)

TMA of 1420 unselected, non-consecutive CRC from 1985 to 1998 was constructed as previously described.[16–21] Normal colonic mucosa samples ($n = 9$, as normal controls) and colonic adenomas ($n = 45$ mild dysplasia, $n = 48$ moderate dysplasia and $n = 48$ severe dysplasia) were also included in this study. The tumours were retrospectively retrieved from the files of the following institutions: Institute of Pathology, University of Basel, Institute of Clinical Pathology, Basel, Institute of Pathology, Stadtsspital Triemli, Zürich, Switzerland.[16–21] One tissue cylinder (0.6 mm diameter) was punched from representative tissue areas (away from the infiltrating tumour border) of each 'donor' tissue block and brought into one recipient block (3×2.5 cm) using a homemade semiautomated tissue arrayer. Three TMA blocks were generated. Clinico-pathological data are reported in Supplementary Table 1.

For IHC analysis standard indirect immunoperoxidase procedures were used (ABC Elite, Vector Laboratories, Burlingame, CA). The antibodies used in this study were raised against the synthetic peptide C-18-R (TVTFREAQAAEGFFRDR) specific for the carboxy-terminal (C-terminal) region of the human CBX7 protein. They were affinity purified against the synthetic peptide.[13] Negative controls were performed by omitting the first antibody and by pre-incubating the first antibody with molar excess of the CBX7 synthetic peptide (1/250, pretreatment: MW 98 °C 30′ in citrate buffer pH6). Further information about TMA construction and IHC are reported in Supplementary Materials and Methods.

2.3. Fluorescence in situ hybridisation (FISH) analysis

For FISH analysis 5 μm sections were treated according to the Paraffin Pretreatment Reagent Kit protocol before hybridisation (Vysis Inc., Abbott Laboratories, Des Plaines, IL). FISH was performed with Spectrum-Green labelled CBX7 probe, each in combination with Spectrum-Orange labelled centromere probe for chromosome 22 (Vysis Inc., Abbott Laboratories). Hybridisation and posthybridisation washes were performed according to the 'LSI procedure' (Vysis Inc., Abbott Laboratories). Slides were then counterstained with 125 ng/ml 4,6-diamino-2-phenylindole in antifade solution. The signals were counted in 25 nuclei per tissue sample. Amplification was defined as a gene probe/centromere probe ratio of $\geqslant 2.0$. Polysomy was defined as an average centromere copy number greater than the average copy number detected in 10 normal control tissues plus 3 SD. Reasons for not evaluable cases were absence of tissue, absence of tumour, not interpretable staining or bad hybridisation.

2.4. Statistical analysis

The association between categorical clinico-pathological features and CBX7 expression was performed using the Chi-square test for categorical variables and with Student's *T*-test for age. Univariate survival analysis was carried out by Kaplan–Meier survival curves and log-rank test, while multivariable analysis was performed by multiple Cox regression analysis, together with pT (pT1 + pT2 versus pT3 + pT4), pN (pN0 versus pN+), MMR-status, tumour location (left versus right), age (>60 years versus <60 years) and vascular invasion. The assumption of proportional hazards was verified by analysing the correlation of Schoenfeld residuals and the ranks of individual failure times. In order to determine the appropriate immunohistochemical cut-off score for CBX7 positivity, receiver operating characteristic (ROC) curve analysis was performed. The sensitivity (true positive) and specificity (false positive) for survival were determined at each possible CBX7 score and the ROC curve was plotted. The (0,1)-criterion was applied to select from the curve the point closest to the

coordinate (0,1) which has the maximum sensitivity and specificity for the outcome. This point was determined to be 50%. Tumours scored as ⩽50% CBX7 expression were therefore classified as negative while those with >50% were denoted as positive. *p*-Values <0.05 were considered statistically significant. Analyses were performed with SAS (SAS Institute Inc., Cary, NC).

2.5. Reverse transcriptase and PCR analysis

Total RNA was extracted from tissues and cell cultures using the RNAeasy Mini Kit (Qiagen, Valencia, CA). One microgram of total RNA from each sample was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen) using an optimised blend of oligo-dT and random primers. PCR was carried out on cDNA using the MyCycler (Biorad, Hercules, CA). The RNA PCR Core Kit (Applied Biosystems, Foster City, CA) was used to perform amplifications. After a first denaturing step (94 °C for 3 min), PCR amplification was performed for 25 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; the primers used are reported in Supplementary Materials and Methods). Negative controls were obtained by carrying out the PCR on samples that were not reverse-transcribed, but otherwise identically processed. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide and scanned with a Typhoon 9200 scanner (GE Healthcare, Piscataway, NJ).

Table 1 – Association of CBX7 (immunohistochemistry) and clinico-pathological features in the whole series of colorectal cancer patients.

Clinico-pathological features		CBX7 + N (%)	*p*-Value
pT	pT1	52 (5.1)	<0.001
	pT2	160 (15.6)	
	pT3	656 (63.9)	
	pT4	159 (15.5)	
pN	pN0	520 (51.5)	0.056
	pN1	276 (27.4)	
	pN2	213 (21.1)	
Grade	G1	22 (2.1)	0.499
	G2	879 (85.4)	
	G3	128 (12.4)	
Vascular invasion	Absent	744 (72.4)	0.035
	Present	284 (27.6)	
Sex	Female	542 (52.0)	0.826
	Male	500 (48.0)	
Tumour location	Left-sided	687 (66.5)	0.044
	Right-sided	346 (33.5)	
MMR status	Proficient	899 (86.3)	0.002
	Deficient	143 (13.7)	
Survival	5-year survival rate	55.1	<0.001[a]
	Median (95%CI)	77 (64–84)	

a Not an independent prognostic factor after adjusting for pT, pN, tumour grade and vascular invasion in multivariable analysis.

2.6. Quantitative real time PCR (qRT-PCR)

To design the assay we used the Human ProbeLibrary™ system (Exiqon, Vedbaek, Denmark) and we chose the primer for CBX7 and G6PD as previously described.[13] qRT-PCR was carried out with the Chromo4 Detector (MJ Research, Waltham, MA) in 96-well plates using a final volume of 20 μl. For PCR we used 8 μl of 2.5× RealMasterMix™ Probe ROX (Eppendorf AG, Hamburg, Germany) 200 nM of each primer, 100 nM probe and cDNA generated from 50 ng of total RNA. Conditions were: 2 min 95 °C; then 45 cycles 20 s 95 °C and 1 min 60 °C. Each reaction was carried out in duplicate. We used the $2^{-\Delta\Delta CT}$ method to calculate relative expression levels.

2.7. Cell culture and transfections

CRC cell lines (HT-29, HCT-116, GEO and CACO-2) were grown in DMEM containing 10% foetal calf serum, glutamine and ampicillin/streptomycin (Gibco Laboratories, Carlsbad, CA) in a 5% CO_2 atmosphere. Cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA). The transfected cells were selected in a medium containing geneticin (G418; Life Technologies, Milan, Italy) and expanded for further analysis.

2.8. Ethics

All the analyses were performed according to the ethical standards of the local ethic committee.

3. Results

3.1. Loss of CBX7 expression correlates with a more aggressive phenotype of CRC

3.1.1. Immunohistochemical analysis of CBX7 expression

A TMA including 1420 cases was analysed by IHC using polyclonal antibodies raised against the C-terminal region of human CBX7 protein. IHC results are summarised in Tables 1 and 2. 1273 cases were evaluable (89.7%): 1042 (81.9%) cases showed at least some degree of positivity. Some representative results of the immunohistochemical analyses are shown in Fig. 1 (Fig. 1A–G). The specificity of the reaction was confirmed by the lack of tissue immunoreactivity after pre-incubation of the antibody with molar excess of the CBX7 synthetic peptide (Fig. 1H) as also previously demonstrated.[13] Negative controls were also performed by omitting the first antibody (data not shown). We used several normal colonic mucosa samples as positive controls ($n = 9$). We found a high degree of positivity for CBX7 staining in all the cases analysed (mean expression 50.0 ± 12.0% of CBX7-positive cells, $p = 0.0003$).

Subsequently, we have evaluated the relationship between CBX7 expression and the following parameters: sex, localisation (left/right), pT, pN, grading and vascular invasion.

3.1.1.1. Whole series. A significant relationship was found with the localisation (negative cases were more frequently observed in the right colon), pT category (higher stage = reduced expression), pN category, vascular invasion and MMR-status. No relationship was observed with grade or sex (Table 1).

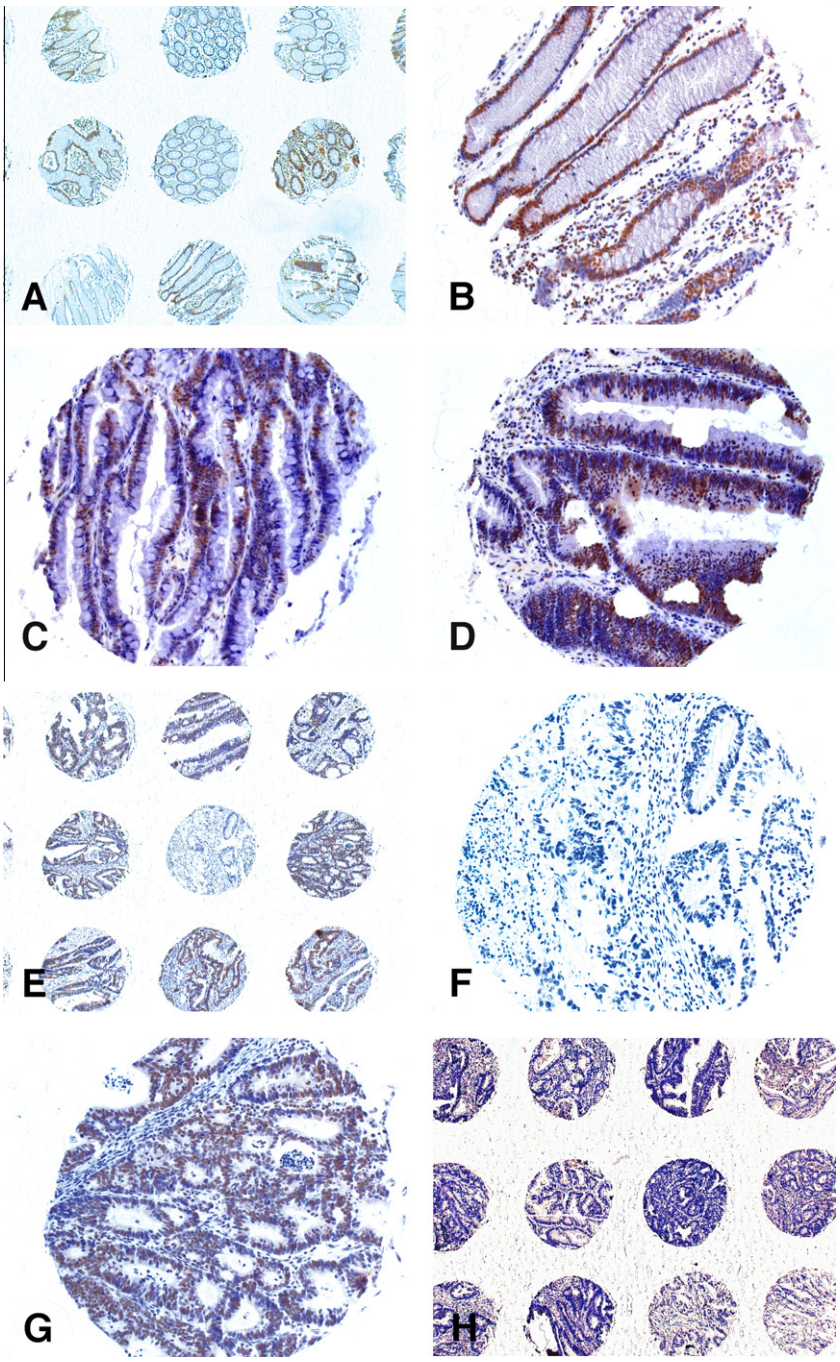

Fig. 1 – Immunohistochemical analysis of a colon cancer TMA for CBX7 protein expression using antibodies raised against a specific CBX7 peptide. (A and B) Intense nuclear immunoreactivity in normal colonic mucosa (A) 5× magnification; (B) 20× magnification. (C) Immunoreactivity in adenoma with mild dysplasia (20× magnification). (D) Immunoreactivity in adenoma with severe dysplasia (20× magnification). (E) Immunoreactivity in colon cancer (5× magnification). (F) Nuclear negativity (20× magnification). (G) Nuclear positivity (20× magnification). (H) No staining in negative control (5× magnification).

3.1.1.2. *MMR-proficient group*. 1080 tumours were evaluable. 899 (83.2%) were immunohistochemically positive for CBX7. A significant relationship was found with pT category ($p < 0.001$), vascular invasion ($p = 0.045$) and localisation ($p = 0.044$) (Table 2).

3.1.1.3. *MMR-deficient group*. 193 tumours were evaluable. There was no significant association with the clinico-pathological characteristics, neither in the IHC analysis nor in the FISH analysis (data not shown).

3.1.1.4. *Survival analysis*. The results are summarised in Supplementary Tables 2 and 3.

3.1.1.5. *Whole series*. In the univariate analysis the immunohistochemical expression of CBX7 was related to survival

Table 2 – Association of CBX7 (immunohistochemistry) and clinico-pathological features in MMR-proficient colorectal cancer patients.

Clinico-pathological features		Positive N (%)	*p*-Value
T stage	pT1	51 (5.8)	<0.001
	pT2	152 (17.2)	
	pT3	552 (62.4)	
	pT4	130 (14.7)	
N stage	pN0	437 (50.4)	0.261
	pN1	246 (28.3)	
	pN2	185 (21.3)	
Tumour grade	G1	22 (2.5)	0.164
	G2	776 (87.4)	
	G3	90 (10.1)	
Vascular invasion	Absent	637 (71.7)	0.045
	Present	251 (28.3)	
Sex	Female	447 (49.7)	0.786
	Male	452 (50.3)	
Tumour location	Left-sided	645 (72.5)	0.044
	Right-sided	245 (27.5)	

($p < 0.001$; Table 1 and Fig. 2A). Stratifying the series according to T and N categories and MMR-status, a significant relationship was found in pT3 ($p = 0.019$; Supplementary Table 2 and Fig. 2B) and in MMR-proficient cases ($p < 0.001$; Supplementary Table 2 and Fig. 3A). In the multivariate analysis no independent prognostic value was found ($p = 0.179$, HR 0.87, 95% confidence interval (CI) 0.7–1.1), as also in MMR-proficient cases ($p = 0.174$, HR 0.86, 95% CI 0.7–1.1).

3.1.1.6. MMR-proficient group. In the univariate analysis the immunohistochemical expression of CBX7 was significantly related to survival in pT3 ($p = 0.012$; Supplementary Table 3 and Fig. 3B) and in pT4N0 ($p = 0.004$; Supplementary Table 3 and Fig. 3C) cases.

3.1.2. Fluorescence in situ hybridisation (FISH) analysis

FISH analysis using a BAC clone covering the CBX7 genomic locus was performed to verify whether it was linked to an allelic loss. The results are summarised in Supplementary Table 4. Then, 959/1420 (67.5%) punches were evaluable. Unexpectedly, monosomy was found only in one case,

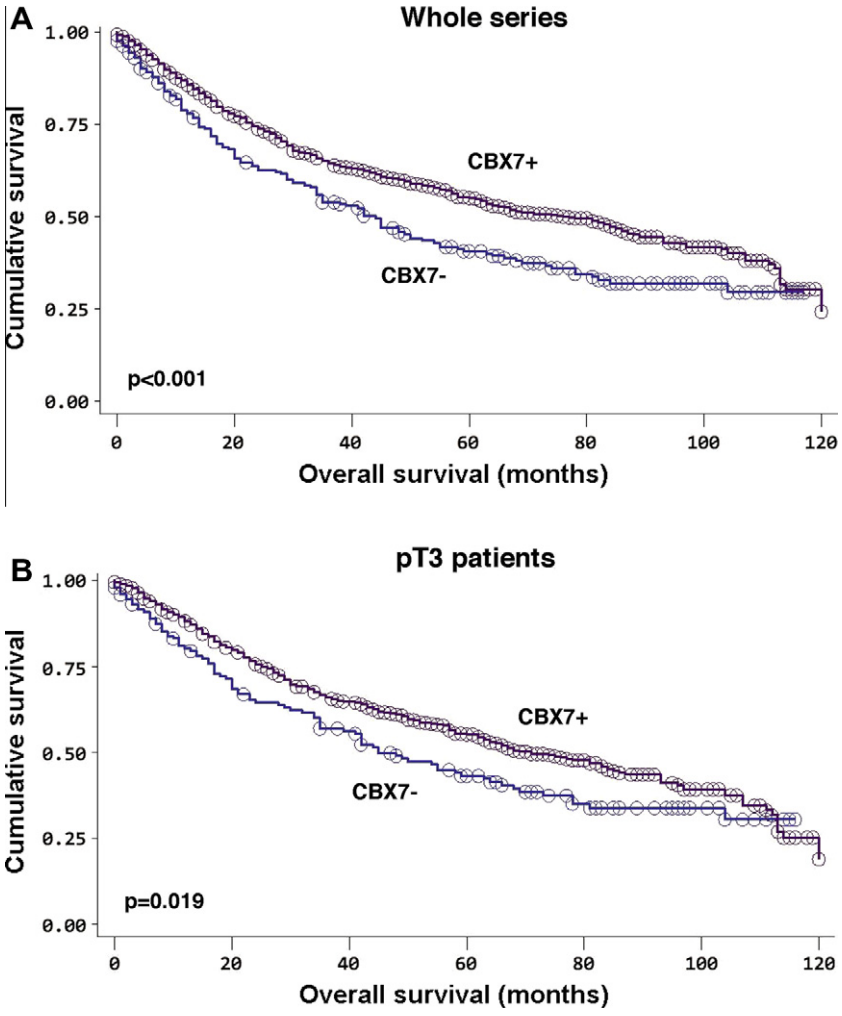

Fig. 2 – IHC Kaplan–Meier survival analysis. (A) Whole series. Tumours showing nuclear positivity for CBX7 showed better survival than cases showing nuclear negativity ($p < 0.001$, log-rank test). (B) pT3 group. Tumours showing nuclear positivity for CBX7 showed better survival than cases showing nuclear negativity ($p = 0.019$, log-rank test).

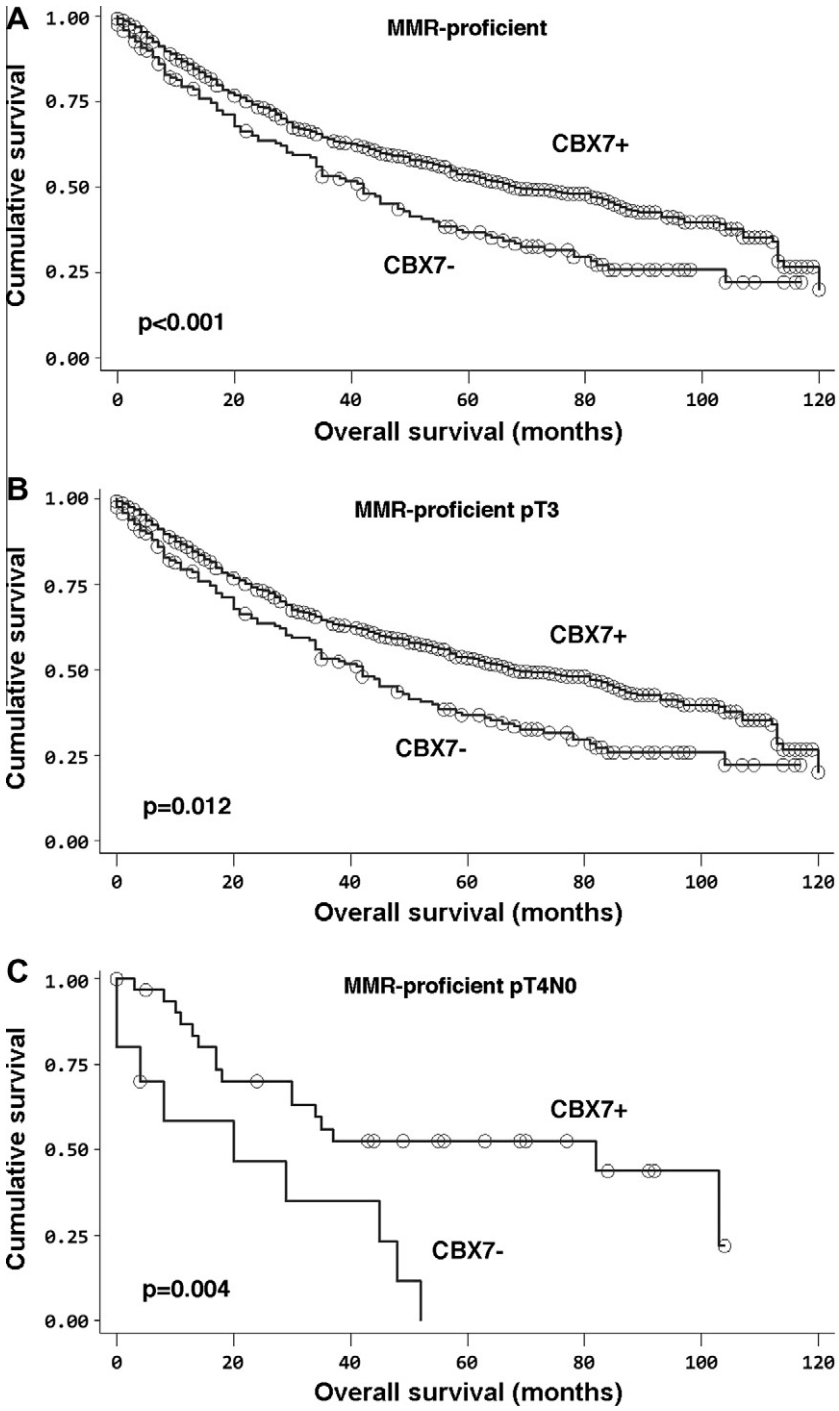

Fig. 3 – IHC Kaplan–Meier survival analysis. (A) MMR-proficient. Tumours showing nuclear positivity for CBX7 showed better survival than cases showing nuclear negativity ($p < 0.001$, log-rank test). (B) MMR-proficient pT3. Tumours showing nuclear positivity for CBX7 showed better survival than cases showing nuclear negativity ($p = 0.012$, log-rank test). (C) MMR-proficient pT4N0. Tumours showing nuclear positivity for CBX7 showed better survival than cases showing nuclear negativity ($p = 0.004$, log-rank test).

whereas a CBX7 polysomy was found in 107 cases (11.2%) and a CBX7 amplification was observed in 14 cases (1.5%). For statistic aims we divided the series into dysomic/monosomic ($n = 838$) versus polysomic/amplified ($n = 121$). No significant relationship was found with clinico-pathological characteristics, as also in MMR-proficient and MMR-deficient cases.

3.1.2.1. Survival analysis. The results are summarised in Supplementary Tables 5 and 6.

3.1.2.2. Whole series. In the univariate analysis the presence of polysomy-amplification was significantly related with better survival ($p = 0.013$; Supplementary Table 4 and Fig. 4A). A

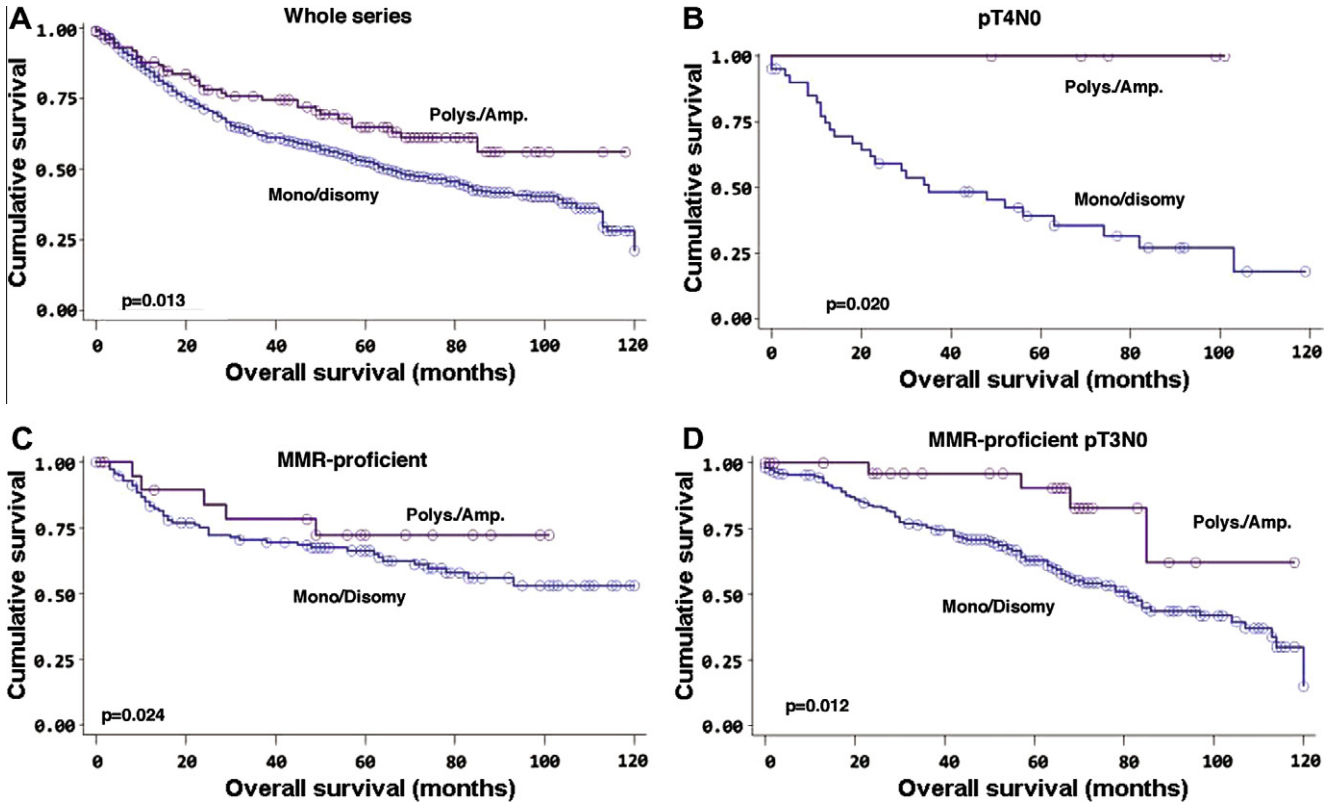

Fig. 4 – Correlation of survival with CBX7 copy number analysed by FISH in CRC patients (Kaplan–Meier survival analysis). (A) Whole series. Tumours showing gain/polysomy at CBX7 locus showed better survival than cases showing disomy/monosomy ($p = 0.013$, log-rank test). (B) pT4N0 group. Tumours showing gain/polysomy at CBX7 locus showed better survival than cases showing disomy/monosomy ($p = 0.020$, log-rank test). (C) MMR-proficient group. Tumours showing gain/polysomy at CBX7 locus showed better survival than cases showing disomy/monosomy ($p = 0.024$, log-rank test). (D) MMR-proficient pT3N0 group. Tumours showing gain/polysomy at CBX7 locus showed better survival than cases showing disomy/monosomy ($p = 0.012$). Univariate survival analysis was carried out by Kaplan–Meier survival curves and log-rank test, while multivariable analysis was performed by multiple Cox regression analysis.

significant relationship was found in the pT4N0 ($p = 0.020$; *Supplementary* Table 5 and Fig. 4B) and pT4N + ($p = 0.003$; *Supplementary* Table 5) subgroups. In the multivariate analysis an independent prognostic value was found ($p = 0.028$, HR 0.67, 95% CI 0.45–0.90).

3.1.2.3. *MMR-proficient group*. In the univariate analysis the presence of polysomy-amplification was significantly related with longer survival ($p = 0.024$; *Supplementary* Table 5 and Fig. 4C). This was true also in pT3 ($p = 0.029$; *Supplementary* Table 6), pT3N0 ($p = 0.012$; *Supplementary* Table 6 and Fig. 4D) and pT4N + ($p = 0.001$; *Supplementary* Table 6) subgroups. No patient in the subgroups pT1, pT2N0 and pT4N0 and showing polysomy-amplification of CBX7 died (*Supplementary* Table 6). The results were still significant for amplified cases ($p = 0.0250$). The multivariate analysis showed an independent prognostic value ($p = 0.023$, HR 0.64, 95% CI 0.40–0.90).

3.2. *CBX7 expression in colonic adenomas*

We have also analysed 141 adenomas ($n = 45$ mild dysplasia, $n = 48$ moderate dysplasia and $n = 48$ severe dysplasia) and found that the mean protein expression of CBX7 decreases when the dysplasia is much more severe (mean expression 29.5 ± 5.4%, 23.1 ± 5.2% and 22.5 ± 5.2%, respectively, % of CBX7-positive cells, $p = 0.0003$). Therefore it seems that the loss of CBX7 expression is positively correlated with the progression of the dysplasia. If we reduce to two groups (normal colonic mucosa + mild dysplasia versus moderate + severe dysplasia) there is also a loss of expression of CBX7 with progression of the dysplasia (33.0 ± 4.9% versus 22.8 ± 3.7%, respectively, $p = 0.0018$).

3.3. *Analysis of CBX7 expression in normal and neoplastic colon tissues by qRT-PCR*

We also evaluated CBX7 expression at mRNA level by qRT-PCR in a panel of matched normal/tumour tissues. A drastic reduction (with a fold-change up to more than 50) in CBX7-specific mRNA levels was observed in CRC samples versus normal counterpart tissue (Fig. 5A). The analysis of CBX7 gene expression in four human CRC cell lines versus normal colonic mucosa by RT-PCR gave rise to similar results (Fig. 5B).

Therefore, these studies suggest that the block of CBX7 expression occurs at a transcriptional level.

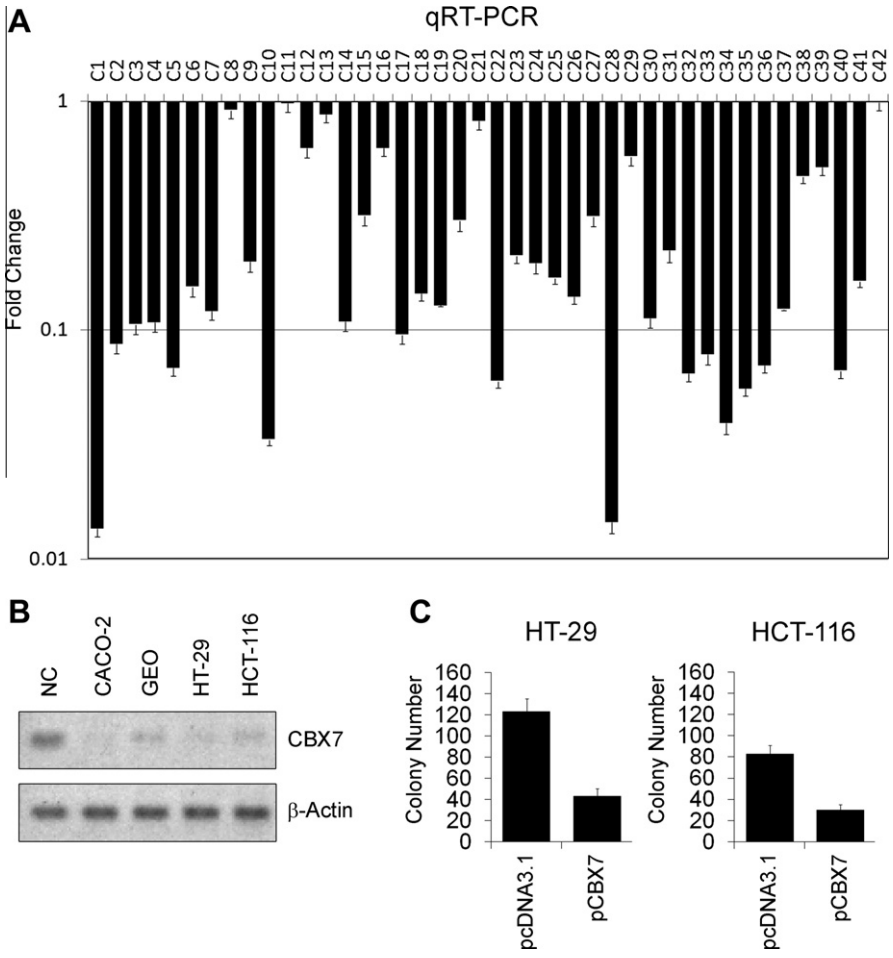

Fig. 5 – (A) Quantitative real time PCR (qRT-PCR) analysis of human CRC samples. The fold-change indicates the relative change in expression levels between tumour samples and normal samples, assuming that the value of each normal sample is equal to 1. CRC, colorectal carcinomas from different patients; NC, normal colonic mucosa. (B) CBX7 gene RT-PCR in human CRC cell lines versus normal colonic mucosa (NC). β-Actin expression served as loading control. (C) Colony-forming assay experiment performed on HT-29 and HCT-116 CRC cell lines transfected with a vector expressing the CBX7 cDNA (pCBX7). The empty vector pcDNA3.1 was used as a control. The reported results are the mean of two experiments and error bars show SD.

3.4. *Restoration of CBX7 gene expression inhibits the growth of colon carcinoma cell lines*

To investigate the role of the loss of CBX7 expression in colon carcinogenesis we analysed the effects of CBX7 restoration in CRC cell lines. To this aim we carried out a colony-forming assay with the CRC cell lines HT-29 and HCT-116 after transfection with the vector carrying the CBX7 gene or the empty backbone vector (Supplementary Materials and Methods). The colonies were scored after 2 weeks. Cells transfected with the CBX7 gene generated a lower (less than 50%) number of colonies in comparison to those observed after cell transfection with the backbone vector (Fig. 5C).

4. Discussion

In this study we have identified, by using TMA technology, IHC and FISH, CBX7 as a prognostic marker for patients with CRC. By IHC performed on normal colonic mucosa, colonic adenomas (moderate, mild and severe dysplasia) and CRC samples, we found that the loss of CBX7 expression is strongly related with the colon cancer phenotype. In fact, we also found that the loss of CBX7 expression is positively correlated with the progression of the dysplasia, as we observed in colonic adenomas. Therefore the loss of CBX7 expression during colorectal carcinogenesis seems to be an early event that predisposes to the following acquisition of a malignant behaviour.

Indeed, here we demonstrate a clear correlation between the loss of CBX7 expression and an advanced pT. A strong relationship was also observed between CBX7 expression and the survival especially in advanced tumours (pT3–pT4), poorer prognosis being associated with lower CBX7 expression. Therefore, CBX7 might identify prognostic subgroups and help to decide the therapeutic strategy.

CRC arises at least through two main oncogenetic pathways. Most of the cases (nearly 85%) show the so-called 'suppressor phenotype', characterised by chromosomal instability and alterations of the WNT pathway.[22,23] The remaining 15% of the cases show the so-called 'mutator phenotype',

characterised by alterations of the MMR-proteins and microsatellite instability. The latter tumours display a peculiar histology, are less aggressive and may have a reduced response to chemotherapy with 5-FU (fluorouracil).[24,25] Therefore, we have decided to stratify our analysis according to MMR-status by performing IHC for MLH-1, MSH-2 and MSH-6. This method has very high sensitivity and specificity and has been validated in our series in several studies.[17,19–21] A significant association of CBX7 expression with clinico-pathological parameters was found only in MMR-proficient cases, thus suggesting that a role of CBX7 in determining the prognosis could be related to the 'suppressor phenotype'.

The correlation of the loss of CBX7 expression with a poor prognosis is not restricted to colon cancer, but it appears to be a more general event in oncology. We have previously demonstrated that CBX7 expression was comparable to normal thyroid tissue in benign thyroid adenomas, slightly reduced in papillary thyroid carcinomas that are well differentiated and slow-growing and completely absent in anaplastic thyroid carcinomas that are completely undifferentiated, very aggressive and always fatal.[13] Moreover, our preliminary results demonstrate the association between a reduced CBX7 expression and a more aggressive histotype also in breast, ovary, pancreas and lung (Troncone et al., manuscript in preparation). The correlation of the loss of CBX7 expression with a poor prognosis could be due to its ability to positively regulate the E-cadherin gene[15] whose loss of expression is a negative prognostic factor for CRC patients.[26,27] Moreover, our preliminary results indicate that CBX7 is also able to negatively regulate the expression of osteopontin, a gene involved in the metastatic process.[28,29] In this picture, our preliminary results also indicate that the restoration of CBX7 expression is able to repress the ability of carcinoma cells to repair a wound in a culture disk as well the migration across the transwell membrane (Pallante et al., manuscript in preparation).

The block of CBX7 expression occurs at mRNA levels since the qRT-PCR analysis of surgically removed CRC confirms the immunohistochemical data. FISH analysis was then performed to verify a possible allelic loss that could account for the lack of CBX7 protein synthesis. Interestingly, monosomy was found only in one case, whereas 107 cases (11.2%) showed CBX7 polysomy and 14 cases (1.5%) showed CBX7 locus amplification. FISH analysis also suggests that the loss of the CBX7 expression is not linked to an allelic loss. Equally, no mutations were detected (data not shown). Therefore, epigenetic mechanisms, such as methylation and chromatin structure, may account for the loss of CBX7 expression. Consistent with this hypothesis, our preliminary data indicate that the HMGA1 proteins that have been demonstrated to be overexpressed in highly malignant neoplasias including colon cancer[30,31] are able to directly downregulate the CBX7 expression.[32]

Here we also confirm that the loss of CBX7 expression plays a role in the process of carcinogenesis since the restoration of CBX7 expression in two CRC cell lines leads to a reduced number of colonies with respect to the same cells transfected with the backbone vector. Flow cytometric analysis suggests that CBX7 may play a critical role in the G1-S phase transition of the cell cycle since this step is slowed down in the presence of the CBX7 protein (data not shown). Consistently, our preliminary results propose the cyclin E, a cyclin involved in the G1-S phase transition, as a target negatively regulated by the CBX7 protein (Federico et al., manuscript in preparation). Moreover, the positive control of E-cadherin expression by CBX7, recently demonstrated by our group,[15] unravels another mechanism by which the loss of CBX7 expression may correlate with a highly malignant phenotype.

It is noteworthy that in apparent contrast with the previous data published by our group[13,15] and those shown here, CBX7 has been also described as an oncogene. Indeed, CBX7 cooperates with c-Myc to produce highly aggressive B-cell lymphomas and can initiate T-cell lymphomagenesis.[9] Moreover, CBX7 extends the lifespan of a wide range of normal human cells and immortalises mouse fibroblasts by downregulating expression of the Ink4a/Arf locus.[33] It is likely that CBX7 may have, as already described for other genes such as E2F and HMGA1[34], oncogenic and tumour suppressor activity, depending on the cellular context. The higher proliferation rate and the increased lifespan of the mouse embryonic fibroblasts (MEFs) null for CBX7 with respect to the wild type MEFs[13] further support our hypothesis.

In conclusion, our study has shown that the loss of CBX7 expression is associated with a poor prognosis in CRC patients and that this gene might be promising in establishing a personalised prognostic and therapeutic approach for CRC patients.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), Progetto Strategico Oncologia Consiglio Nazionale delle Ricerche, the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MIUR). This work was supported from NOGEC-Naples Oncogenomic Center. We thank the Associazione Partenopea per le Ricerche Oncologiche (APRO) for its support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.05.011.

REFERENCES

1. Stewart BW, Kleihues P, editors. *World cancer report*. Lyon: IARC Press; 2003.
2. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;**87**:159–70.
3. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *N Engl J Med* 2003;**348**:919–32.

4. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004;**10**:789–99.
5. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005;**23**:609–18.
6. Liu W, Liu Y, Zhu J, et al. Reduced hGC-1 protein expression is associated with malignant progression of colon carcinoma. *Clin Cancer Res* 2008;**14**:1041–9.
7. Eschrich S, Yang I, Bloom G, et al. Molecular staging for survival prediction of colorectal cancer patients. *J Clin Oncol* 2005;**23**:3526–35.
8. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. *Cell* 2007;**128**:735–45.
9. Scott CL, Gil J, Hernando E, et al. Role of the chromobox protein CBX7 in lymphomagenesis. *Proc Natl Acad Sci USA* 2007;**104**:5389–94.
10. Wu JI, Lessard J, Crabtree GR. Understanding the words of chromatin regulation. *Cell* 2009;**136**:200–6.
11. Bernstein E, Duncan EM, Masui O, et al. Mouse polycomb proteins bind differentially to methylated histone H3 and RNA and are enriched in facultative heterochromatin. *Mol Cell Biol* 2006;**26**:2560–9.
12. Lund AH, van Lohuizen M. Polycomb complexes and silencing mechanisms. *Curr Opin Cell Biol* 2004;**16**:239–46.
13. Pallante P, Federico A, Berlingieri MT, et al. Loss of the CBX7 gene expression correlates with a highly malignant phenotype in thyroid cancer. *Cancer Res* 2008;**68**:6770–8.
14. Hinz S, Kempkensteffen C, Christoph F, et al. Expression parameters of the polycomb group proteins BMI1, SUZ12, RING1 and CBX7 in urothelial carcinoma of the bladder and their prognostic relevance. *Tumour Biol* 2008;**29**:323–9.
15. Federico A, Pallante P, Bianco M, et al. The CBX7 protein, whose expression is decreased in human carcinomas, positively regulates E-cadherin expression by interacting with the HDAC2 protein. *Cancer Res* 2009;**69**:7079–87.
16. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998;**4**:844–7.
17. Lugli A, Zlobec I, Minoo P, et al. Role of the mitogen-activated protein kinase and phosphoinositide 3-kinase/AKT pathways downstream molecules, phosphorylated extracellular signal-regulated kinase, and phosphorylated AKT in colorectal cancer-a tissue microarray-based approach. *Hum Pathol* 2006;**37**:1022–31.
18. Tornillo L, Lugli A, Zlobec I, et al. Prognostic value of cell cycle and apoptosis regulatory proteins in mismatch repair-proficient colorectal cancer: a tissue microarray-based approach. *Am J Clin Pathol* 2007;**127**:114–23.
19. Zlobec I, Baker K, Terracciano LM, Lugli A. RHAMM, p21 combined phenotype identifies microsatellite instability-high colorectal cancers with a highly adverse prognosis. *Clin Cancer Res* 2008;**14**:3798–806.
20. Zlobec I, Terracciano L, Tornillo L, et al. Role of RHAMM within the hierarchy of well-established prognostic factors in colorectal cancer. *Gut* 2008;**57**:1413–9.
21. Zlobec I, Vuong T, Compton CC, et al. Combined analysis of VEGF and EGFR predicts complete tumour response in rectal cancer treated with preoperative radiotherapy. *Br J Cancer* 2008;**98**:450–6.
22. Grady WM, Markowitz SD. Genetic and epigenetic alterations in colon cancer. *Annu Rev Genomics Hum Genet* 2002;**3**:101–28.
23. Jass JR. Molecular heterogeneity of colorectal cancer: implications for cancer control. *Surg Oncol* 2007;**16**:S7–9.
24. Kim GP, Colangelo LH, Paik S, et al. Predictive value of microsatellite instability-high remains controversial. *J Clin Oncol* 2007;**25**:4857.
25. Kim GP, Colangelo LH, Wieand HS, et al. Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study. *J Clin Oncol* 2007;**25**:767–72.
26. Roca F, Mauro LV, Morandi A, et al. Prognostic value of E-cadherin, beta-catenin, MMPs (7 and 9), and TIMPs (1 and 2) in patients with colorectal carcinoma. *J Surg Oncol* 2006;**93**:151–60.
27. Ngan CY, Yamamoto H, Seshimo I, et al. A multivariate analysis of adhesion molecules expression in assessment of colorectal cancer. *J Surg Oncol* 2007;**95**:652–62.
28. Agrawal D, Chen T, Irby R, et al. Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. *J Natl Cancer Inst* 2002;**94**:513–21.
29. Yeatman TJ, Chambers AF. Osteopontin and colon cancer progression. *Clin Exp Metastasis* 2003;**20**:85–90.
30. Chiappetta G, Manfioletti G, Pentimalli F, et al. High mobility group HMGI(Y) protein expression in human colorectal hyperplastic and neoplastic diseases. *Int J Cancer* 2001;**91**:147–51.
31. Fusco A, Fedele M. Roles of HMGA proteins in cancer. *Nat Rev Cancer* 2007;**7**:899–910.
32. Mansueto G, Forzati F, Ferraro A, et al. Identification of a new pathway for tumor progression: microRNA-181b upregulation and CBX7 downregulation by HMGA1 protein. *Genes and Cancer* 2010. doi:10.1177/1947601910366860.
33. Gil J, Bernard D, Martinez D, Beach D. Polycomb CBX7 has a unifying role in cellular lifespan. *Nat Cell Biol* 2004;**6**:67–72.
34. Fedele M, Fidanza V, Battista S, et al. Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice. *Cancer Res* 2006;**66**:2536–43.