BCL2L1 has a functional role in colorectal cancer and its protein expression is associated with chromosome 20q gain

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Abstract

Colorectal cancer (CRC) is the second leading cause of cancer death in the western world. The majority of CRCs, which develop from adenoma precursor lesions, show gain of chromosome arm 20q, where BCL2L1 is located. BCL2L1 is an important apoptosis regulating gene that codes for both an anti-apoptotic (Bcl-xL) and a pro-apoptotic (Bcl-xS) splice variant. The aim of the present study was to investigate whether BCL2L1 contributes to 20q gain-driven colorectal adenoma-to-carcinoma progression. To this end, the functional role of BCL2L1 in cancer-related processes was investigated, and differences in BCL2L1 DNA, mRNA, and protein levels were compared between colorectal adenomas and CRCs, as well as between tumours with and without 20q gain. Down-modulation of BCL2L1 inhibited cell viability and anchorage-independent growth of CRC cells, while invasion was not affected. BCL2L1 DNA copy number and protein expression were increased in CRCs compared to adenomas (\( p = 0.00005 \) and \( p = 0.03 \), respectively), while mRNA expression was not. Differences in BCL2L1 protein expression were even more pronounced between tumours with and without 20q gain (\( p = 0.0001 \)). In conclusion, BCL2L1 is functionally involved in several cancer-related processes and its protein expression is associated with 20q gain. This supports a role for 20q gain-dependent expression of BCL2L1 in colorectal adenoma-to-carcinoma progression. However, the absence of a direct correlation between BCL2L1 mRNA and protein expression implies that BCL2L1 protein expression is regulated at the post-transcriptional level by a distinct factor on the 20q amplicon (eg ZNF217, AURKA or miRNAs). Therefore, even though BCL2L1 affects CRC biology in a 20q gain-dependent manner, it is not likely to be a driver of chromosome 20q gain associated adenoma-to-carcinoma progression.
**Introduction**

Colorectal cancer (CRC) is the third most common tumour type in men and women in the western world. After lung cancer, CRC ranks second in terms of cancer deaths [1]. CRC evolves from adenoma precursor lesions. The development of adenomas from normal colon epithelium is initiated by disruption of the WNT signalling pathway. Progression of pre-malignant adenoma lesions to carcinomas takes place in only about 5% of cases [2]. Two major pathways contribute to adenoma-to-carcinoma progression: microsatellite instability (MSI) and chromosomal instability (CIN). MSI is observed in approximately 15% of CRCs and is caused by a failing DNA mismatch repair system, resulting in the accumulation of DNA mutations. The majority of CRCs (about 85%) show CIN, meaning that parts of or whole chromosomes are gained or lost. CIN can result in loss of tumour suppressor genes or amplification of oncogenes, but also in copy number changes of miRNA loci, leading to aberrant expression [3,4].

Gain of chromosomal region 20q is frequent in CRC and has been associated with colorectal adenoma-to-carcinoma progression [5]. Furthermore, gain of 20q correlates with poor prognosis in CRC patients [6]. Chromosome 20q harbours several putative oncogenes, including AURKA, BCL2L1, SRC, and ZNF217 [5,7-10]. The BCL2L1 locus is among the loci with the highest copy number ratios [10].

BCL2L1 (BCL2-like 1) belongs to the family of Bcl-2 proteins that are involved in the regulation of the mitochondrial pathway of apoptosis by controlling the release of pro-apoptotic factors from the mitochondrion. This protein family comprises pro- and anti-apoptotic members, which are all characterized by Bcl-2 homology domains [11]. The mitochondrial pathway of apoptosis is stimulated by several triggers such as DNA damage and cytotoxic drugs. Upon these signals, the pro-apoptotic Bcl-2 proteins are activated and when their activation levels pass a certain threshold, the inhibitory effects of anti-apoptotic Bcl-2 family members are overcome. Pro-apoptotic Bcl-2 proteins change the mitochondrial membrane permeability, which results in the release of pro-apoptotic factors including cytochrome c. Cytochrome c in combination with pro-caspase-9 and Apaf-1 form the apoptosome, which activates pro-caspase-9 by a conformational change. Caspase-9 subsequently causes activation of downstream executor caspases which execute apoptosis (Figure 1) [11,12].

BCL2L1 pre-mRNA can give rise to several splice variants, of which the long Bcl-xL isoform and the short Bcl-xS isoform are the most important. These two isoforms differ in size and function. Bcl-xL is 233 amino acids in length and has anti-apoptotic effects, whereas Bcl-xS, which lacks 63 amino acids compared with Bcl-xL, is pro-apoptotic. Bcl-xS heterodimerizes with Bcl-xL and inhibits its anti-apoptotic effects [11,13]. The balance between the Bcl-xL and Bcl-xS isoforms is regulated by transcriptional events and alternative splicing [14]. Several growth factors (eg IL-1α, IL-6, and GM-CSF) and apoptosis signals (eg Fas and chemotherapeutic drugs) can shift splicing of BCL2L1 pre-mRNA in favour of Bcl-xS [15,16].

Bcl-xL mRNA is overexpressed in CRCs compared with normal tissues [17]. Protein expression of BCL2L1 has been reported as being increased in about 60% of CRCs compared with normal epithelium, and in 50% of colorectal adenomas [8]. Overexpression of Bcl-xL contributes to apoptosis resistance, resulting in improved survival of malignant cells, which contributes to the development of tumour metastasis and a poor response to chemotherapy [18,19]. However, data on the functional consequences of BCL2L1
overexpression on cancer-related processes in CRC are limited. One study has reported that down-regulation of BCL2L1 expression inhibited anchorage-independent growth and invasion in a CRC cell line [19].

The aim of the present study was to investigate whether BCL2L1 may drive chromosome 20q amplification in colorectal adenoma-to-carcinoma progression by investigating the role of BCL2L1 in cancer-related processes in CRC cell lines. In addition, BCL2L1 DNA, mRNA, and protein levels were studied in colorectal adenomas and CRCs and in relation
to chromosome 20q gain, since genes that drive 20q gain-associated colorectal cancer progression are expected to show a correlation between DNA copy number, mRNA expression, and protein expression.

Materials and methods

Collection, storage, and use of tissue and patient data were performed in agreement with the ‘Code for Proper Secondary Use of Human Tissue in the Netherlands’, in compliance with national and institutional ethical regulations.

Cell culture

Three human CRC cell lines with gain of chromosomal region 20q were selected: Caco2, HT29 and SW480 (Supporting information, Supplementary Figure 1). HT29 and SW480 cells were cultured in DMEM (Lonza BioWhittaker, Verviers, Belgium) containing 10% fetal bovine serum (FBS) (HyClone, Perbio Science, Etten-Leur, The Netherlands). Caco2 cells were grown in RPMI1640 (Lonza BioWhittaker) containing 20% FBS. Cell culture media were supplemented with 2 mM L-glutamine, 100 IU/ml sodium-penicillin, and 100 mg/ml streptomycin. The cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂.

siRNA transfection

*BCL2L1* mRNA expression was down-regulated using a pool of four small interfering RNA (siRNA) (SMARTpool M-003 458-00 from Dharmacon Inc, Lafayette, CO, USA). Transfections were performed according to manufacturer’s recommendations to obtain a final siRNA concentration of 30 nM. A Non-Targeting control siRNA pool (Non-Targeting Pool #2; D-001 206-14) was used as a negative control. Down-modulation of *BCL2L1* expression was confirmed by qRT-PCR (Supporting information, Supplementary materials and methods).

Cell viability assay

siRNA transfections were performed in triplicate in 96-well flat bottom plates using a siRNA pool directed against polo-like kinase 1 (*PLK1*; M-003 290-01; Dharmacon) as a positive control. Cells were left untreated for 3 days (regular culture conditions) or were treated with 5FU (5-fluoro-deoxyuridine; Sigma-Aldrich, Zwijndrecht, The Netherlands) at a final concentration of 2 μM (Caco2 and SW480) or 3 μM (HT29) for 5 days (5FU-induced cytotoxicity). The number of viable cells was measured using the MTT assay.

Anchorage-independent growth assay

SW480 cells were transfected in duplicate in 24-well cell culture plates using siPLK1 as a positive control. Cells from one well were cultured in 0.35% SeaPlaque agarose (Lonza, Verviers, Belgium) in 10% FBS-containing medium, in duplicate. The MTT cell viability assay was performed on the second well to correct the number of observed colonies by the number of viable cells. After 3 weeks of incubation, the number of colonies was evaluated. Digital images were taken and colony numbers were counted using image processing.
**Invasion assay**

SW480 cells were transfected in duplicate in 12-well cell culture plates. *BIRC5* down-modulation has previously been described as inhibiting invasion of SW480 and was used as a positive control [20]. Transfected cells were transferred to extracellular matrix gel (ECM gel; Sigma-Aldrich)-coated transwells containing a fluorescence-blocking filter (HTS FluoroBlok; Falcon, BD Biosciences, Alphen aan de Rijn, The Netherlands). Invasion was stimulated for 48 h by a FBS gradient and analysed by fluorescent quantification of invaded as well as non-invaded cells using calcein-AM (Molecular Probes, Invitrogen, Leiden, The Netherlands). Ratios of invaded versus non-invaded cells were calculated.

**Normalization of functional data**

Log$_2$ ratios of *BCL2L1* and controle genes relative to siNon-Targeting were calculated per assay. Data from the respective assays were normalized to correct for day-to-day variation and to reduce the effect of technical or biological outliers using robust regression (R package MASS, function ‘rlm’, default settings). This was achieved using data from *BCL2L1* and 35 other genes (Sillars-Hardebol et al, accepted for publication).

**BCL2L1 immunohistochemical analysis on tissue microarrays**

Immunohistochemical staining of BCL2L1 was performed on tissue microarrays composed of 82 colorectal adenomas and 82 CRCs with up to three cores (0.6 mm) per tumour. Histological and molecular characteristics of the tumours, including microsatellite instability and chromosome 20q gain status, are described in Supporting information, Supplementary Table 1. In addition, BCL2L1 staining was performed on siRNA-treated SW480 cells to verify the effect of siBCL2L1 on protein expression (Supporting information, Supplementary materials and methods). MAB4625 (Millipore Corporation, Billerica, MA, USA) antibody recognizing both Bcl-x$_S$ and Bcl-x$_L$ was incubated for 30 min at room temperature (1:100 dilution). Staining was detected by HRP-coupled polymer (ThermoScientific, Warm Springs, Fremont, CA, USA) and visualized by diaminobenzidine plus (DAB Plus) (ThermoScientific). Incubation without primary antibody was used as a negative control.

Tumours were scored for BCL2L1 protein expression based on the staining intensity of epithelial cells as weak, moderate or strong. The maximum score of the evaluated cores was determined per tumour. For statistical analysis, tumours were classified into two groups based on the maximum staining score: weak and moderate staining versus strong staining.

**Statistical analysis**

Data on the DNA copy number ratio of the actual *BCL2L1* locus and mRNA expression in colorectal adenomas and CRCs have been previously obtained by array CGH (comparative genomic hybridization) and mRNA expression microarrays, respectively [5]. Differences in the copy number ratio and mRNA expression between groups of tumours (adenomas and CRCs or tumours with and without 20q gain) were assessed by the Mann-Whitney test. Correlation of array CGH data and mRNA expression data was evaluated by the Pearson correlation. The chi-square test was used to compare protein expression between groups of tumours. Correlation of protein expression with the DNA.
copy number ratio (CGH data) or mRNA expression was evaluated by the Mann-Whitney test. Statistical analyses were performed in SPSS (version 15.0 for Windows; SPSS, Chicago, IL, USA).

More details about the materials and methods may be found in the Supporting information, Supplementary materials and methods.

Results

**BCL2L1 affects cell viability and anchorage-independent growth**

The role of BCL2L1 in CRC carcinogenesis was investigated by analysing the functional effects of BCL2L1 down-regulation on cancer-related processes. BCL2L1 mRNA expression was down-regulated using a siRNA pool which efficiently reduced BCL2L1 mRNA (five-fold) and protein expression (Figure 2A and 2B). First, the effect of BCL2L1 down-regulation on cell viability was investigated. Figure 2C shows a two-fold reduction of Caco2 and SW480 cell viability, but no change in HT29 cell viability under regular culture conditions. After induction of cellular cytotoxicity with 5FU, a drug commonly used in the treatment of CRC patients, the cell viability of all three cell lines was greatly reduced.

Other important characteristics of malignant cells are survival and proliferation, independent of surrounding cells, as well as cellular invasion into underlying tissue. To evaluate these features in vitro, we performed anchorage-independent growth in soft agarose and invasion assays. While all three colorectal cancer cell lines were suited to monitor cell viability, the SW480 cell line clearly outperformed Caco2 and HT29 with respect to anchorage-independent growth and invasion capacity, thereby providing a much better window of opportunity to monitor siRNA-mediated inhibition of these assays. BCL2L1 down-modulation resulted in an almost four-fold reduction in the number of colonies formed in soft agarose (Figure 2D). However, BCL2L1 mRNA down-modulation did not inhibit invasive capacity, in contrast to the positive control BIRC5 (Figure 2E).

**BCL2L1 DNA copy number, mRNA and protein expression**

The DNA copy number ratio of BCL2L1 was analysed using an array CGH dataset of 34 colorectal adenomas and 33 CRCs [5]. The BCL2L1 DNA copy number was significantly increased in CRCs relative to adenomas (Mann-Whitney: \( p = 0.00005 \)) (Figure 3A). The mRNA expression levels of BCL2L1 were compared in a microarray expression dataset of 68 colorectal tumours; 37 adenomas, and 31 CRCs [5]. Colorectal adenomas and carcinomas did not differ in BCL2L1 mRNA expression (Mann-Whitney: \( p = 0.5 \)) (Figure 3B). Immunohistochemistry performed on tissue microarrays containing 82 colorectal adenomas and 82 colorectal carcinomas was used to determine protein expression levels of BCL2L1. BCL2L1 was expressed in the cytoplasm of epithelial cells, in general with a stronger staining intensity in the supranuclear zone (Figure 4). Expression of BCL2L1 protein was significantly higher in CRCs than in adenomas (chi-square: \( p = 0.03 \)) (Figure 3C).

**BCL2L1 protein expression is associated with chromosome 20q gain**

Gain of chromosomal region 20q, at which BCL2L1 is located, plays an important role in colorectal adenoma-to-carcinoma progression. Chromosome 20q gain status was de-
determined based on the overall level of DNA copy number alterations along the chromosome arm, which may differ from the BCL2L1 DNA copy number status that was based on probes at the BCL2L1 locus. The association between 20q gain and BCL2L1 protein expression was examined by comparing BCL2L1 protein expression in microsatellite-stable tumours with and without 20q gain. Tumours (adenomas and carcinomas) with 20q gain had significantly higher expression of BCL2L1 compared with tumours without 20q gain (chi-square: \( p = 0.0001 \)). Separate analysis of adenomas and carcinomas demonstrated that adenomas with 20q gain have significantly higher BCL2L1 protein expression than...
Figure 3 BCL2L1 DNA copy number, mRNA expression, and protein expression in human colorectal adenoma and carcinoma tissues. (A) DNA copy number ratio (log2 ratio) of BCL2L1 in an array CGH dataset of 34 adenoma and 33 carcinoma samples. p values were calculated by the Mann-Whitney test. (B) BCL2L1 mRNA expression levels (arbitrary units) in a microarray expression dataset of 37 colorectal adenomas and 31 carcinomas. p values were calculated by the Mann-Whitney test. (C) Protein expression of BCL2L1 on tissue microarrays composed of 82 colorectal adenoma and 82 carcinoma samples. For statistical analysis, tumours were classified into two groups based on the maximum staining score: weak and moderate staining versus strong staining. p values were determined by the chi-square test.

Figure 4 Immunohistochemical staining examples of BCL2L1. BCL2L1 staining was predominantly cytoplasmic, generally associated with stronger supranuclear staining. The staining intensity of epithelial cells was scored as weak, moderate or strong. Examples of each category are shown.
adenomas without 20q gain (chi-square: $p = 0.005$), while such a significant difference was not observed for carcinomas (Figure 5A).

Although there was no difference in BCL2L1 mRNA expression in carcinomas compared with adenomas (Mann-Whitney: $p = 0.5$) (Figure 3B), tumours with 20q gain did show a trend towards increased BCL2L1 mRNA expression compared with tumours without 20q gain ($p = 0.09$) (Figure 5B). Separate analysis for adenomas with and without chromosome 20q gain also revealed a trend towards increased mRNA expression ($p = 0.1$) (Figure 5B). A similar comparison could not be made for carcinomas because mRNA data were only available for two carcinomas without 20q gain. DNA copy number ratios at the BCL2L1 locus itself did not correlate with BCL2L1 mRNA expression levels (Pearson correlation: $R = 0.13$ with $p = 0.3$) (Figure 6A). These observations therefore do not support a gene-dosage effect for BCL2L1. In addition, BCL2L1 DNA copy number ratios and mRNA expression levels did not correlate with protein expression (Figure 6B and 6C).

**Discussion**

Gain of chromosome 20q is a major factor in colorectal adenoma-to-carcinoma progression. Genes that drive 20q gain-associated colorectal cancer progression are expected to affect carcinogenesis-related processes and to show a correlation between DNA copy number, mRNA expression, and protein expression. One of the oncogenes within the 20q amplicon is BCL2L1, an apoptosis regulating gene. In the present study, BCL2L1 was found to contribute to cell viability and anchorage-independent growth of CRC cells, which implies a functional role for BCL2L1 in the biology of CRC.

In order to identify whether BCL2L1 may drive chromosome 20q amplification in colorectal adenoma-to-carcinoma progression, the levels of BCL2L1 DNA, mRNA, and protein were compared between adenomas and carcinomas and in relation to chromosome 20q gain. BCL2L1 protein expression was investigated using an antibody that recognizes the
BCL2L1 in association with 20q gain

Bcl-xL as well as the Bcl-xS isoform. In positive cases, BCL2L1 immunohistochemistry typically showed strong supranuclear staining against a weaker overall cytoplasmic staining. This is consistent with previously reported staining patterns of BCL2L1 and Bcl-xL [23,24]. The anti-apoptotic Bcl-xL mRNA is most abundantly expressed in several tissue types in mice [25]. Also in human (cancer) tissues, mRNA (as detected by RT-PCR) and protein (as detected by western blotting) expression levels of Bcl-xL are higher than those of Bcl-xS, which often do not reach the detection level [26,27]. Although we observed both Bcl-xL and Bcl-xS transcripts by RT-PCR in tumour samples and in the SW480 cell line, relatively high levels of Bcl-xS were only present in SW480 cells upon 5FU-induced cytotoxicity (Supporting information, Supplementary Figure 2). Therefore, the observed BCL2L1 protein expression probably primarily represents Bcl-xL, which corresponds to the oncogenic features of BCL2L1 in terms of copy number gain and its role in cancer processes. Previously, higher Bcl-xL protein expression has been found in CRCs and adenomas compared with normal tissue [17]. In the present study, we show that protein expression of BCL2L1 was stronger in CRCs than in colorectal adenomas. So, while in the present series of colorectal tumours BCL2L1 protein expression was associated with overall 20q gain, it did not correlate with the DNA copy number status of the actual BCL2L1 locus or with its mRNA expression. This is consistent with observations in endometrium, where also no correlation was found between BCL2L1 mRNA and protein expression levels [27]. Since BCL2L1 mRNA expression levels did not correlate with either BCL2L1 locus DNA copy status or BCL2L1 protein expression, alternative mechanisms must explain the 20q-related change in protein expression. For instance, BCL2L1 protein expression could be regulated at the post-transcriptional level by a distinct factor on 20q. Indeed, protein products of other genes and miRNAs at 20q are important in post-transcriptional regulation of gene expression. Interestingly, the mRNA expression of ZNF217, another putative oncogene on chromosome 20q, did correlate with BCL2L1 protein expression and may therefore contribute to BCL2L1 post-transcriptional regulation (Supporting information, Supplementary Figure 3). In addition, AURKA (located on 20q13.2), a cell-cycle regulated kinase involved in the assembly of the mitotic spindle and which contributes to carcinogenesis, has recently been found to regulate BCL2L1 mRNA splicing [28]. Our understanding of the role of miRNAs in the regulation of mRNA translation is still in

Figure 6 Correlation of BCL2L1 DNA copy number and mRNA and protein expression. (A) Correlation of BCL2L1 DNA copy number with mRNA expression. Pearson correlation coefficient and p value are shown. Lines represent a linear regression fit with 95% confidence intervals. (B) Correlation of BCL2L1 DNA copy number with protein expression. p values were calculated by the Mann-Whitney test. (C) Correlation of BCL2L1 mRNA expression with protein expression. p values were calculated by the Mann-Whitney test.
its infancy. Although much has been revealed about the mechanism by which miRNAs regulate translation, a major challenge remains to identify the mRNA targets of miRNAs. Several miRNAs have been predicted to regulate \( BCL2L1 \) expression, including miR-663, miR-1289-1, miR-296, miR-298, miR-646, miR-647, and miR-133a-2, which are located on chromosome arm 20q [29]. A possible role of these miRNAs in regulating \( BCL2L1 \) protein expression remains to be resolved.

Previously, gain of the 20q amplicon has been correlated with poor prognosis of CRC patients [6]. Since in the present study \( BCL2L1 \) protein expression is linked to 20q gain, \( BCL2L1 \) may contribute to this effect. A correlation between increased \( BCL2L1 \) expression and poor prognosis has been found in CRC [24] and is most probably caused by disruption of apoptosis pathways. Chemotherapeutic drugs act by inducing apoptosis; for example, they shift the splicing of \( BCL2L1 \) pre-mRNA in favour of pro-apoptotic \( Bcl-x_s \) [16]. Functional apoptosis pathways are therefore important determinants of a successful therapy response. Although chemotherapeutic drugs improve CRC patient survival, resistance to these treatments arises, leading to therapy failure and, ultimately, death of the patient. Of the multiple factors that contribute to chemotherapy resistance, overexpression of the anti-apoptotic \( Bcl-x_L \) isoform of \( BCL2L1 \) is a major one [30-32]. Several inhibitors of \( Bcl-x_L \) with pro-apoptotic and anti-carcinogenic effects have been identified [33-35]. The use of these inhibitors on their own or combined with chemotherapeutic drugs could be a useful strategy to modify intrinsic drug resistance in patients with high \( BCL2L1 \) expression.

In conclusion, \( BCL2L1 \) contributes to cancer-related processes and its protein expression is associated with chromosome 20q gain. This is consistent with a role for \( BCL2L1 \) in 20q gain-associated colorectal adenoma-to-carcinoma progression. \( BCL2L1 \) itself, however, is not a likely driver of chromosome 20q gain since the DNA copy number status of the \( BCL2L1 \) locus does not correlate with \( BCL2L1 \) mRNA and protein expression levels. Therefore, protein expression of \( BCL2L1 \) is anticipated to be regulated at the post-transcriptional level by a distinct factor on the 20q amplicon (e.g., \( ZNF217 \), \( AURKA \) or miRNAs).

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