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Molecular cloning of novel alternatively spliced variants of *BCL2L12*, a new member of the *BCL2* gene family, and their expression analysis in cancer cells

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ABSTRACT

In the past, we identified and cloned the *BCL2-like 12 (BCL2L12)* gene, a novel member of the *BCL2* family, which is implicated in various malignancies. The classical BCL2L12 protein isoform contains a highly conserved BH2 domain, a BH3-like motif, and a proline-rich region, and is involved in apoptosis. Most members of this apoptosis-related family are subjected to alternative splicing, thus generating multiple protein isoforms with distinct properties, and sometimes even with opposite function (pro- vs. anti-apoptotic). In the current study, we report the identification, molecular cloning, and expression pattern of novel splice variants of the human *BCL2L12* gene in cancer cell lines. EST clones displaying high sequence identity (≥90%) with the classical *BCL2L12* transcript were aligned, in order to identify those containing at least one novel splice junction. EST database mining led to the identification of three previously unknown splice variants of this apoptotic gene. In our effort to experimentally validate these novel transcripts, we also cloned seven more, previously unidentified, *BCL2L12* alternatively spliced variants. Expression analysis of all *BCL2L12* splice variants in human cancer cell lines and embryonic kidney cells revealed remarkable differences between their *BCL2L12* expression profiles. Interestingly, 7 out of 10 novel splice variants of *BCL2L12* are predicted to encode new protein isoforms, some of which are BH3-only proteins, in contrast to the classical BCL2L12 isoform, which also contains a functional BH2 domain. The remaining three novel splice variants of *BCL2L12* are nonsense-mediated mRNA decay (NMD) candidates.

1. Introduction

BCL2L12 is a newly identified member of the BCL2 family of apoptosis-related genes. Currently, three distinct transcripts resulting from alternative splicing of the *BCL2L12* gene are known. The largest splice variant (*BCL2L12* v.1) consists of seven coding exons and its translation produces the classical BCL2L12 protein isoform (BCL2L12 is.1), a 334-amino acid (aa) polypeptide containing a highly conserved BH2 domain, a BH3-like motif, and a proline-rich region (Kontos et al., 2009; Scorilas et al., 2001). Expression of the full-length mRNA transcript has been observed in many tissues, including breast, thymus, prostate, fetal liver, colon, placenta, pancreas, small intestine, spinal cord, kidney, and bone marrow. An alternative splice variant lacking exon 3 and designated as *BCL2L12-A* (or *BCL2L12* v.2) is mainly expressed in fetal liver, spinal cord, and skeletal muscle (Scorilas et al., 2001). Furthermore, the sequence of a third *BCL2L12* splice variant (*BCL2L12* v.3) that makes use of an alternate in-frame splice site at the 5′ end of exon 3, compared to the full-length transcript, has been deposited in GenBank. The resulting isoform (BCL2L12 is.3) has the same N- and C-termini compared to the main isoform, but is shorter by 1 aa (Thomadaki and Scorilas, 2006).

Data about the localization of the BCL2L12 protein seem to be confusing at the moment. Initially, this protein was detected both in cytosol and mitochondria (Toumelin et al., 2006), yet Stegh et al. (2007) reported that BCL2L12 protein localization is predominantly cytosolic and nuclear without demonstrable mitochondrial association, in human astrocytes and glioma cells. Other studies have shown that both BCL2L12 and BCL2L12-A isoforms are mainly localized to the nucleus of various human cell lines (HeLa, MCF-7, MDA-MB-231, and 293 T cells) (Hong et al., 2010), unlike other members of the BCL2 family, which predominantly localize to cytoplasm and

Abbreviations: BCL2L12, BCL2-like 12; BH2 domain, BCL2-homology-2 domain; BH3-like motif, BCL2-homology-3-like motif; HSPB5, heat shock protein beta-5; UV, ultraviolet; aa, amino acid(s); HSP70, heat shock 70-kDa protein; DFS, disease-free survival; OS, overall survival; NPC, nasopharyngeal carcinoma; CLL, chronic lymphocytic leukemia; EST(s), expressed sequence tag(s); cDNA, DNA complementary to RNA; CO_2, carbon dioxide; FBS, fetal bovine serum; ku, kilo-units; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; oligo(dT), oligodeoxythymidine; DTT, dithiothreitol; dNTP, deoxyribonucleoside triphosphate; u, unit(s); RNase, ribonuclease; PCR, polymerase chain reaction; UTR, untranslated region; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EtdBr, ethidium bromide; ORF, open reading frame; PXXP motif, Pro-any-any-Pro motif; nt, nucleotide(s); 3D, three-dimensional; PTC, premature translation termination codon; NMD, nonsense-mediated mRNA decay; BH1 domain, BCL2-homology-1 domain; BH4 domain, BCL2-homology-4 domain; CDKN1A, cyclin-dependent kinase inhibitor 1A; ARE, AU-rich element.

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mitochondria (Jia et al., 1999). However, Nakajima et al. showed that the mouse Bcl2l12 protein, detected in both the cytoplasm and nucleus, was notably concentrated in the perinuclear region of embryonic fibroblasts, and more precisely in the Golgi apparatus rather than in mitochondria (Nakajima et al., 2009).

Although it is clear that BCL2L12 is involved in apoptosis, it remains somewhat obscure or even controversial whether its role is pro- or anti-apoptotic (Thomadaki and Scorilas, 2006). Mechanistically, unlike typical BCL2 family proteins, BCL2L12 does not affect cytochrome c release or apoptosome-driven caspase-9 activation, but instead it is likely to inhibit post-mitochondrial apoptosis signaling at the level of effector caspase activation, in primary murine cortical astrocytes and human glioma cell lines (Stegh et al., 2007). In fact, BCL2L12 obstructs directly caspase-7 processing, possibly through protein–protein interaction, and indirectly caspase-3 maturation, potently through a remarkable upregulation of the small heat-shock protein α-basic crystallin (αB-crystallin/HSPB5) (Stegh et al., 2008b). By antagonizing effector caspases 3 and 7 downstream of mitochondrial membrane disintegration, BCL2L12 shifts the cell death balance from apoptosis to necrosis (Stegh et al., 2008a). Besides that, nuclear BCL2L12 interacts with the tumor suppressor protein p53 and impedes the capacity of this latter to bind some of its target gene promoters. Thus, BCL2L12 attenuates endogenous p53-directed transcriptomic changes following DNA damage and inhibits p53-dependent senescence and apoptosis processes in glioma cells (Stegh and Depinho, 2011; Stegh et al., 2010). However, in mouse embryonic fibroblasts Bcl2l12 functions as a pro-apoptotic factor upon genotoxic stress, sensitizing UV-irradiated cells to apoptosis (Toumelin et al., 2006). The reason for the seemingly contradictory data between different studies may be a species-specific functional difference between human and mouse full-length BCL2-like 12 isoforms, as the human BCL2L12 protein has an additional 84-aa peptide at the N-terminus, compared with the mouse Bcl2l12 protein. Interestingly, this N-terminal sequence contains a nuclear localization signal, which has been suggested as being responsible for nuclear localization of human BCL2L12 and BCL2L12-A proteins in some cell lines (Hong et al., 2010). The N-terminal 120-aa peptide contains also a sequence responsible for interaction of these proteins with HSP70, which protects them from N-terminal ubiquitination and subsequent proteasomal degradation (Yang et al., 2009).

Expression analysis of *BCL2L12* demonstrated increased expression of both transcripts of this gene in colon cancer samples compared to their normal counterparts (Kontos et al., 2008a; Mathioudaki et al., 2004). Furthermore, colon cancer patients overexpressing *BCL2L12* had significantly longer disease free survival (DFS) and overall survival (OS) (Kontos et al., 2008a). High mRNA expression of *BCL2L12* has also been linked with favorable outcome in patients with breast cancer, since *BCL2L12*-positive patients had a lower probability of relapse and/or death, compared to *BCL2L12*-negative patients (Talieri et al., 2003; Thomadaki et al., 2007). Additionally, it has been suggested that *BCL2L12* could serve as a favorable biomarker in gastric cancer, with significant prognostic impact for DFS and OS (Florou et al., 2010). Recently, *BCL2L12* mRNA expression has also been linked to unfavorable prognosis in nasopharyngeal carcinoma (NPC) and has been suggested as a novel,

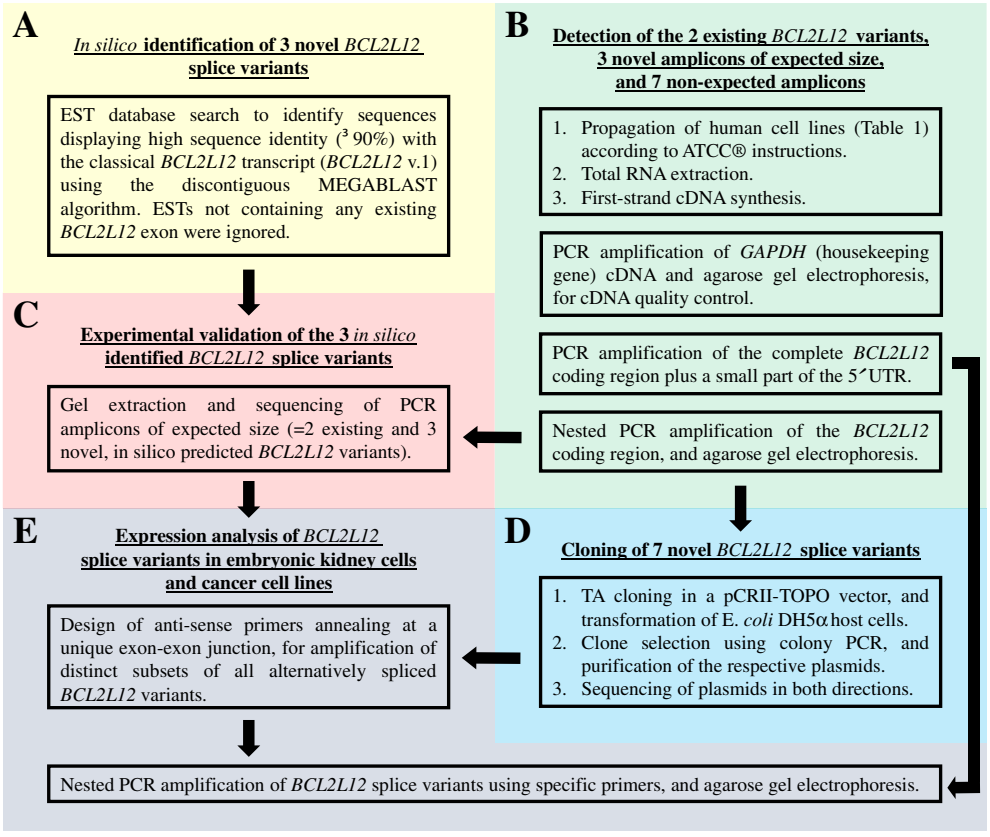

Fig. 1. Flow chart presenting step-by-step the experimental procedure that was followed in the current study. (A) *In silico* analysis of EST clones showing high sequence identity (≥90%) with the classical *BCL2L12* transcript and containing a complete open reading frame (ORF) revealed three novel *BCL2L12* splice variants. (B) In order to validate experimentally these novel *BCL2L12* splice variants in human cells, embryonic kidney cells and a panel of cancer cell lines originating from multiple human tissues were used. Nested PCR amplification of the complete coding region of the *BCL2L12* cDNA and subsequent agarose gel electrophoresis uncovered the existence of multiple distinct PCR amplicons. (C) The three amplicons of size matching the size of the *in silico* identified *BCL2L12* variants were extracted from agarose gel and sequenced, (D) while seven non-expected bands were subcloned, and the respective plasmids were then sequenced. Thus, seven novel *BCL2L12* splice variants were experimentally identified. (E) Expression analysis of all *BCL2L12* alternatively spliced transcripts was performed in the subcultured human embryonic kidney cells and cancer cell lines.