Coxsackie and Adenovirus Receptor Promotes Adenocarcinoma Cell Survival and Is Expressionally Activated after Transition from Preneoplastic Precursor Lesions to Invasive Adenocarcinomas

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Abstract

Purpose: The cell adhesion protein, coxsackie and adenovirus receptor (CAR), is differentially expressed in various human adenocarcinomas. We analyzed the role of differential CAR expression during tumorigenesis and in cell survival of adenocarcinomas.

Experimental Design: In a murine mammary cancer model, a syngenic preneoplastic mammary tissue was implanted into the mammary fat pads of syngenic female BALB/c mice. CAR expression was determined by semiquantitative reverse transcription-PCR in the preneoplastic noninvasive precursor lesions and the developing invasive adenocarcinomas. Cell clones overexpressing CAR were generated and tested for their response to apoptotic factors and for the expression of apoptosis relevant proteins by reverse transcription-PCR and Western blot analysis.

Results: In comparison of preneoplastic precursor lesions with established adenocarcinomas, CAR expression was enhanced 2- to 5-fold in all six tissues which had survived and transformed into invasive adenocarcinomas. When stable CAR-overexpressing cell clones of the human cancer cell lines HeLa, CaSki, and A2780 were compared with the parental cell lines, 1.5- to 6-fold more cells survived application of tumor necrosis factor – related apoptosis-inducing ligand or growth factor withdrawal. CAR-enhanced cell survival was accompanied by reduced activation of caspase 3 and enhanced expression of bcl-2 or bcl-XL, depending on the cell type tested. Upregulation of bcl-2 was found in all CAR-expressing adenocarcinomas of the murine cancer model.

Conclusions: CAR expression is enhanced after transition from preneoplastic precursor lesions to neoplastic mammary cancer outgrowths. Enhanced CAR expression can promote cancer cell survival. These data suggest differential expression of CAR as a new factor in tumorigenesis.

The coxsackie and adenovirus receptor (CAR; refs. 1–3) is a cell-cell adhesion protein in normal epithelia cells (4, 5) as well as in human cancer cells (6). When studying the expression level of CAR in adenocarcinomas of various tissues, it has been repeatedly observed that CAR expression was reduced in less differentiated cancer tissues (7–9). In contrast to these observations, a recent study has shown that CAR expression is necessary for the formation of human lung cancer xenografts in mice (10). Furthermore, enhanced CAR expression has been observed in metastatic prostate cancer cells (9). Thus, by differential expression, CAR could be involved in processes necessary for cancer progression such as cell adhesion and cell survival.

In this study, we analyzed the expression level of CAR in a mouse model of mammary tumorigenesis and found enhanced expression of CAR in all invasive adenocarcinomas. For a better understanding of the effect of enhanced CAR expression in cancer cells, we investigated CAR-expressing cell clones of two widely available human adenocarcinoma cell lines and one squamous cell carcinoma cell line. To test the potential function of CAR in cell survival of cancer cells, CAR-overexpressing cell clones from the A2780 ovarian cancer cell line and the cervical cancer cell lines CaSki and HeLa were tested for their response to apoptotic stimuli, such as the tumor necrosis factor – related apoptosis-inducing ligand (TRAIL), serum withdrawal, or irradiation by UV light.

Materials and Methods

Cell lines and cell culture. Cervical squamous carcinoma cell line CaSki (ATCC CRL-1550), cervical adenocarcinoma cell line HeLa (ATCC CCL-2), and ovarian adenocarcinoma cell line A2780 (ECACC 93112519) are widely distributed, commercially available cell lines. Cells were cultured in DMEM supplemented with 10% FCS, 100 μg streptomycin per mL, 100 units penicillin G per mL, and 2 mmol/L glutamine at 37°C in a humidified atmosphere with 5% CO_2. All cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany).

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Establishing of coxsackie and adenovirus receptor–expressing cell clones. Establishing of CAR-V5–overexpressing cell clones of A2780 and CaSki cells has recently been described (11). CAR-V5–overexpressing HeLa cells were established accordingly.

Induction of apoptosis. A total of 5×10^4 cells was seeded in 24-well cell culture chambers. After 24 hours of incubation, apoptosis was induced by addition of 50 ng/mL TRAIL (Peprotech, London, United Kingdom), irradiation with UV light (254 nm) in a UV cross-linker (LTF Labtech, Wasserburg, Germany) with a setting of 10 mJ, or by changing the DMEM supplemented with 10% FCS against FCS-free DMEM. After 24 hours of further incubation, the number of viable cells was calculated by trypan blue exclusion using a hemocytometer. All experiments were done in duplicate.

Determination of caspase activity. A total of 1×10^6 cancer cells was seeded in 24-well cell culture dishes and allowed to grow for 48 hours. Cells were then incubated for 3 hours with or without 50 ng/mL TRAIL and lyzed in 300 μL cell culture lysis buffer [25 mmol/L Tris-phosphate (pH 7.8), 2 mmol/L DTT, 2 mmol/L 1,2-diaminocyclohexane-tetraacetic acid, 1% Triton X-100, and 10% glycerol]. After centrifugation, 100 μL of the supernatant was incubated with 1 μL of 100 mmol/L colorimetric Ac-DEVD-pNA Caspase-3-Substrat I (Calbiochem, La Jolla, CA) for 3 hours at 37°C. Color development was analyzed at 405 nm in a microtiter reader and related to the amount of protein determined in the corresponding cell extract.

Western blot analysis. Western blot analysis was done as described (12). For antigen detection, a monoclonal anti-V5 antibody (Invitrogen), monoclonal anti–bcl 2 (clone 100) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-bcl X antibody (PharMingen, BD Bioscience, Heidelberg, Germany), and a monoclonal anti–β-actin antibody (Sigma, Schnelldorf, Germany) was used.

Reverse transcription-PCR analysis. RNA preparation and reverse transcriptase reaction was essentially as described (6). Amplification of a 439-bp murine CAR fragment was by using primer pair AATGTGACCAACCTGCAGCT/AGCACAAGGGCCAGCAGCGT, of a 379-bp murine bcl-2 fragment by using primer pair CTGACCCTCCGCCGGGCTGG/AGCAGGGTCTTCAGAGCAG, and of a 369-bp murine bcl-XL fragment by using primer pair CAGCTTCACATAACCCCAGG/ACTTCCGACTGAAGAGTGAG. As an internal standard, amplification of a 340-bp fragment of the murine large ribosomal protein cDNA was done using primer pair GTTCCTGAGGAACATGCGCT/TTCACAGGGGCCTGGGCACC.

Tissues. The TM preneoplastic outgrowth lines developed after transplantation of established chemically transformed mouse mammary epithelial cell lines (MMEL) into cleared mammary fat pads of 3-week-old syngenic BALB/cMed mice (13). In this model system, preneoplastic outgrowth lines were serially transplanted and maintained for extended periods of time (up to 12 months) in the mammary fat pads of syngenic female BALB/c mice. The tissues were removed either as preneoplasias at 8 to 12 weeks after transplantation (TM2L) or as tumors 5 to 7 months after transplantation (tumors T5839, T4032, T4676, T3227, T4404, and T4031). After removal, the preneoplastic outgrowths and primary invasive adenocarcinomas were frozen at −80°C for further analysis.

Results

Coxsackie and adenovirus receptor expression is increased in invasive primary adenocarcinomas. We used an *in vivo* mouse model of mammary tumorigenesis to study CAR expression in preneoplastic precursor lesions and their corresponding primary adenocarcinomas that finally had evolved from the precursor lesions. Murine CAR expression was analyzed by semiquantitative reverse transcription-PCR analysis (Fig. 1A) and normalized against expression of murine large ribosomal protein as an internal standard (Fig. 1B). In the noninvasive preneoplasias (TM2L), CAR expression was low (Fig. 1A). CAR expression was strongly increased in all of the six invasive adenocarcinomas generated from the precursor lesions (Fig. 1A). When related to the expression of the housekeeping gene *LRP*, CAR expression was increased in adenocarcinomas in the range of 2- up to 5-fold (Fig. 1B).

Coxsackie and adenovirus receptor expression can enhance resistance against apoptosis. Stable CAR-overexpressing cell clones A2780CAR6 and CaSkiCAR1 were established from the ovarian cancer cell line A2780 and the cervical cancer cell line CaSki using a V5-tagged human CAR expression plasmid, as previously reported by us (11). These exogenous CAR-V5–expressing cell clones were tested in comparison with their parental cell lines for their response against a variety of apoptotic stimuli, including TRAIL, irradiation by UV light (UV-C, 254 nm), or serum deprivation (Fig. 2). The number of surviving cells was calculated 48 hours after application of the apoptotic stimulus. Figure 2A and B shows that after treatment with TRAIL, UV light, or serum withdrawal, the number of viable cells was higher in cell clones with CAR being overexpressed. The effect varied upon the different apoptotic stimuli applied, resulting in a 1.5-fold higher cell number of A2780CAR6 cells under conditions of TRAIL treatment and in a 6-fold enhanced number of A2780CAR6 cells under serum deprivation conditions (Fig. 2A). CAR-overexpressing CaSki-CAR1 cells revealed a 2- to 3-fold higher number of surviving cells under application of TRAIL or UV radiation (Fig. 2B).

Similar results, showing enhanced resistance against TRAIL-mediated apoptosis, were obtained when other CAR-V5–expressing cell clones of the CaSki cell line or the HeLa cell

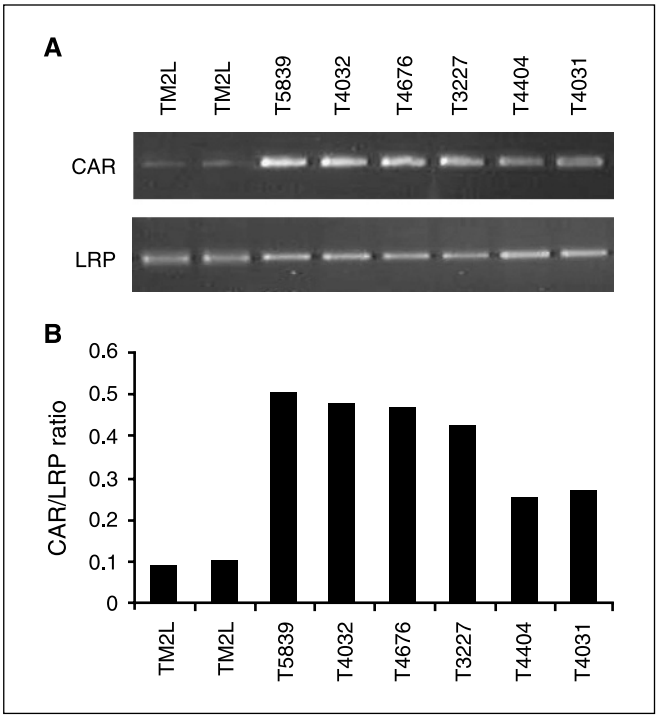

Fig. 1. CAR expression is upregulated in invasive adenocarcinomas in comparison to their precursor noninvasive lesions. *A*, semiquantitative reverse transcription-PCR analysis of preneoplastic lesions TM2L and six different invasive adenocarcinomas developed out of the TM2L lesion (T 5929, T 4032, T 4676, T 3227, T 4404, T 4031) was done using primers specific for murine CAR and large ribosomal protein (*LRP*) as an internal standard. *B*, CAR and large ribosomal protein levels as determined in (*A*) were analyzed by the AIDA image analysis program (Raytest) and expressed as CAR/LRP ratios.

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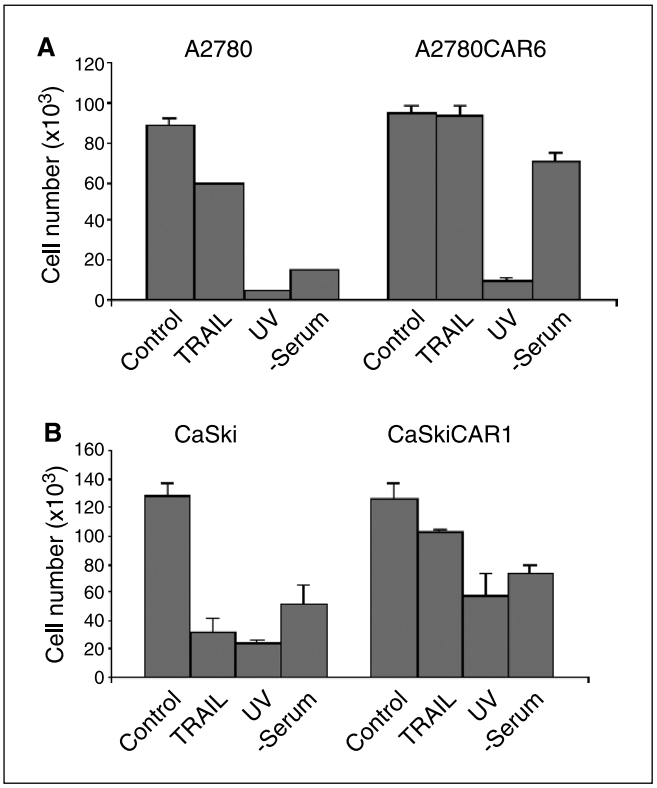

Fig. 2. CAR expression mediates enhanced resistance against various apoptotic factors. A total of 5×10^4 cells of the indicated cell lines was seeded in 24-well cell culture wells and treated as indicated with 5 ng/mL TRAIL, 10 mJ UV-C light (UV cross-linker), or kept in serum-free DMEM. The number of viable cells was counted after 48 hours by using a hemocytometer.

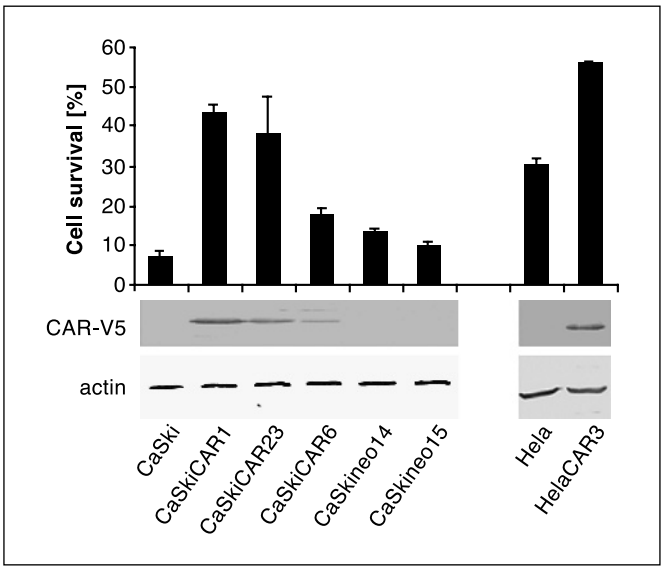

Fig. 3. Antiapoptotic effect of CAR-V5 expression is related to the expression level of CAR-V5. CaSki cell clones with differential expression levels of CAR-V5 as well as HeLa and CAR-V5–expressing HeLaCAR3 cells were established. Expression levels of CAR-V5 are shown by Western blot analysis (*bottom*). A total of 5×10^4 cells of the indicated cell clones in 24-well cell culture plates was treated with or without 5 ng/mL TRAIL and the percentage of cells surviving application of TRAIL was calculated (*columns*).

line were tested and compared with their parental cell lines or neomycin-resistant counterparts (Fig. 3).

TRAIL is a highly effective inducer of apoptosis, resulting in activation of specific cell degrading caspases. Treatment of parental CaSki cells with TRAIL at 5 ng/mL induced cell death in nearly 80% to 90% of all TRAIL-treated cells (Figs. 2 and 3). Apoptosis was concomitted with activation of caspase 3, as shown in Fig. 4. In CAR-overexpressing CaSkiCAR1 cell clones, application of TRAIL induced significant less activation of caspase 3 (Fig. 4).

Coxsackie and adenovirus receptor expression mediates up-regulation of bcl-2 and bcl-XL. It has previously been shown that reexpression of integrin adhesion proteins in Chinese hamster ovary cells can exert a cell-protective effect by up-regulation of bcl-2 (14). For elucidation of the molecular mechanism behind the antiapoptotic effect of CAR expression in human cancer cells, Western blot analysis of the antiapoptotic bcl family members bcl-2 and bcl-XL was done. In the CAR-V5–expressing ovarian cancer cell line A2780CAR6, up-regulation of bcl-2 was observed (Fig. 5). No significant up-regulation of bcl-2 was observed in the CAR-overexpressing cervical carcinoma cell clones CaSkiCAR1 or HeLaCAR3. In these cell lines, Western blot analysis revealed enhanced expression of bcl-XL in the CAR-overexpressing cell clones (Fig. 5).

Up-regulation of bcl-2 in invasive adenocarcinomas. Up-regulation of bcl family members in CAR-overexpressing cell clones *in vitro* raised the question whether bcl-2 and bcl-XL are expressionally regulated in CAR-expressing adenocarcinomas *in vivo*. In the preneoplastic TM2L tissue, bcl-2 expression was rather low but increased significantly (3- to 4-fold) in all tested adenocarcinomas (Fig. 6A and B). The expression level of bcl-XL was found either unchanged or only moderately increased in the tested adenocarcinomas (Fig. 6A and C).

Discussion

The functional role of differential CAR expression in cancer and in particular in adenocarcinomas has not been established yet. CAR expression is frequently found reduced in invasive adenocarcinomas including prostate, esophageal, colorectal, ovarian, and non–small cell lung cancer (7, 9, 12, 15, 16).

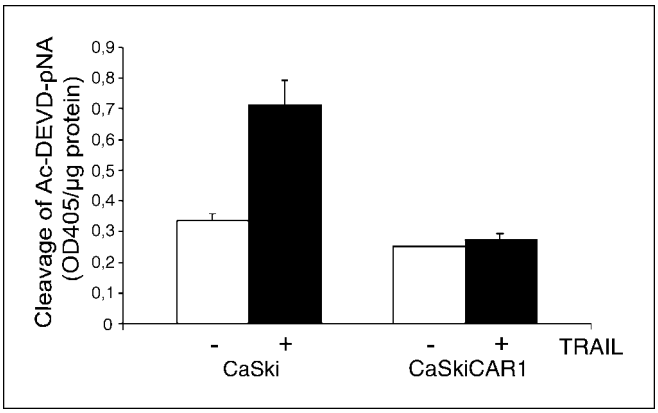

Fig. 4. Reduced Caspase-3 activity in CAR-V5–expressing CaSkiCAR1 cells. CaSki and CaSkiCAR1 cells were incubated for 3 hours with or without 50 ng/mL TRAIL and analyzed for caspase 3 enzymatic activity by using colorimetric caspase 3 substrate Ac-DEVD-pNA. Caspase activity was calculated as $A_{405\ nm}$ related to the amount of protein of the corresponding cell extract.

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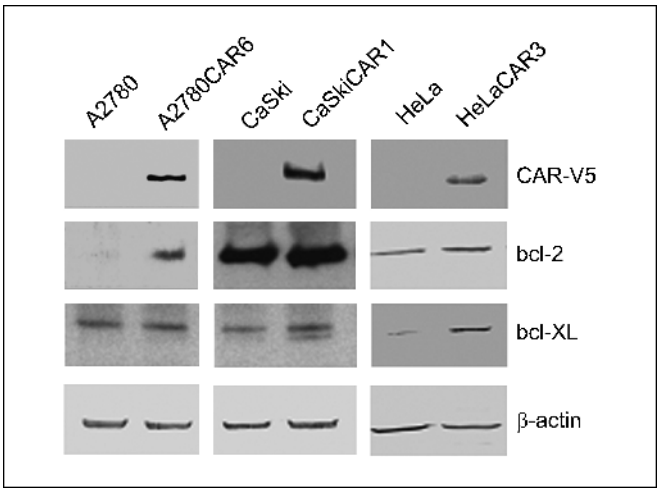

Fig. 5. Regulation of apoptosis-modulating factors in CAR-expressing cell clones. The indicated cell clones were analyzed by Western blot analysis for the expression levels of bcl-2, bcl-XL, CAR-V5 and β-actin.

However, expression of CAR was found necessary for the formation of lung adenocarcinoma xenografts in mice, as lung cancer cells were shown to lose their tumorigenic potential through antisense-mediated down-regulation of CAR (10). We here show that invasive adenocarcinomas expressed CAR at a higher level than the preneoplastic murine mammary tissue they developed from. Earlier studies using this mouse cancer model found a number of distinct changes in the expression of nuclear splicing factors during the transformation from preneoplastic to invasive lesions (17). The mouse mammary tumor model has been well characterized as a multistep tumor system. The outgrowths are clonal cell populations, as previously shown in several molecular analyses (18–20). Enhanced CAR expression in adenocarcinoma outgrowths as described here and the involvement of CAR in adenocarcinoma xenograft formation (10) suggests a potential function of CAR in early development of at least adenocarcinomas. The protective effect of enhanced CAR expression against endogenously produced death receptor ligands (TRAIL) or serum deprivation, as described in this study, could contribute to the neoplastic progression of adenocarcinomas.

In nonmalignant tissues, several localization studies for CAR revealed enrichment of CAR at basolateral cell-cell adhesions and tight junctions of untransformed epithelial cells (4, 5). In cancer cells, which in general lose epithelial organization, still enrichment of CAR at cell-cell adhesions has previously been described by us (6). Several cell adhesion proteins are not only functioning as structural proteins but can also function as "adhesion-activated signaling receptors" mediating cellular signaling processes as described for integrins (21) and cadherins (22). Binding of cell adhesion proteins to extracellular matrix proteins or to other homotypic or heterotypic cell adhesion proteins of neighboring cells can mediate cellular survival signals. Loss of these survival signals by cellular detachment can induce cell death. In Chinese hamster ovary cells, an integrin-dependent up-regulation of bcl-2 has been described (14). We found in this study that CAR expression in A2780, CaSki, and HeLa cells increased cancer cell survival, associated with enhanced bcl-2 and/or bcl-XL expression, depending on the cell line tested. In the murine mammary cancer model, we observed significant up-regulation of bcl-2 expression in all tested, CAR-overexpressing adenocarcinomas *in vivo*. An effect of CAR expression on other antiapoptotic or proapoptotic pathways can not be excluded yet. Changes in the expression level of bcl family members could be a direct effect of CAR expression mediated by yet unidentified signaling pathways or a secondary effect mediated by enhanced cell-cell adhesions and thus activation of other signaling pathways. Whereas the effect of TRAIL is primarily executed by specific induction of apoptosis, the cytoprotective effect of CAR expression against UV irradiation and serum withdrawal suggest additional, still unidentified cell protective functions of CAR expression in cancer cell survival.

The role of CAR in the metastatic process has not been clarified yet. The study that described reduced CAR expression in regionally advanced prostate cancer observed enhanced CAR expression in metastatic prostate cancer cells (9). Similar observations of up-regulation at metastatic sites have recently been made also for the expression of cadherins (23, 24), primarily regarded as tumor suppressor proteins and often down-regulated during cancer progression (25, 26). Up-regulation of CAR in metastatic cancer cells could either support reattachment at the metastatic site or mediate enhanced survival of cancer cells in transit. The above-cited

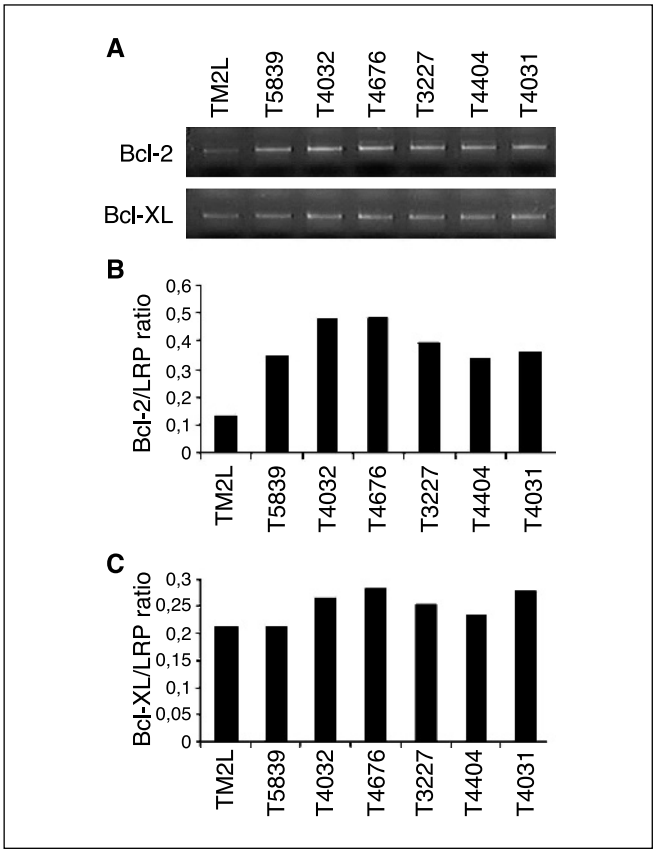

Fig. 6. Enhanced bcl-2 expression in invasive adenocarcinomas. *A*, semiquantitative reverse transcription-PCR analysis for bcl-2 and bcl-XL expression of the preneoplastic lesion TM2L and six different invasive adenocarcinomas as introduced in Fig. 1 was done. *B* and *C*, Bcl-2, or bcl-XL, as indicated and analyzed in (*A*), and large ribosomal protein (*LRP*) levels as determined in Fig. 1*A*, were analyzed by the AIDA image analysis program (Raytest) and expressed as bcl/LRP ratios.

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analysis of CAR expression in prostate cancer showed enhanced CAR expression in all tested (four of four) lymph node metastases (9). Lymphocytes, either present in the lymphatic system or the interstitial cancer environment, produce significant amounts of secreted or cell membrane-bound death receptors, such as tumor necrosis factor, FasL, or TRAIL (27). Thus, enhanced CAR expression could at least in adenocarcinomas increase the resistance of cancer cells against apoptosis-inducing attacks by the immune system in early stages of tumorigenesis as well as during a metastatic process.

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