1 Introduction

Laser capture microdissection (LCM) is a powerful methodology that allows us to retrieve specific types of normal and diseased cells from tissue sections. The dissected tissue sections can then be used for DNA, RNA or protein expression analysis. Thus, in accordance with the recent LCM technological/methodological advancement, LCM also has been applied to tumor angiogenesis research.

Angiogenesis is the growth of new blood vessels from pre-existing vasculature in response to interactions between tumor cells and endothelial cells, growth factors, and extracellular matrix components. Tumor angiogenesis is an absolute requirement for tumor growth and development. In recent years, the effect of Bcl-2 (B-cell lymphoma 2) expression on endothelial cells during tumor angiogenic events is one that is recognized. First, Bcl-2 is shown to be upregulated in endothelial cells exposed to VEGF and that upregulation of Bcl-2 in these cells is sufficient to enhance tumor angiogenesis and tumor growth. More recently, it has been demonstrated that Bcl-2 functions as a pro-angiogenic signaling molecule in endothelial cells through a pathway that involves activation of the canonical NF-kB pathways resulting in the upregulation of angiogenic chemokines CXCL1 and CXCL8. Moreover, Bcl-2 also activates the STAT3 signaling pathway in endothelial cells resulting in the upregulation of VEGF secretion and induction of Bcl-2 expression in tumor cells via VEGFR1. Thus, the effect of Bcl-2 expression on endothelial cells during tumor angiogenic events becomes more importance.

To quantify the Bcl-2 expression on tumor-associated endothelial cells, LCM is also a powerful methodology. Thus, in the present review, we focus on the presentation and discussion of existing literature in the field of Bcl2 and tumor angiogenesis, and especially provide an overview of the field of tumor angiogenesis research assisted with LCM. In addition, we present a brief protocol of cDNA synthesis following immune-LCM from paraffin-embedded tissue sections.

2 The Bcl-2 Family

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2.1 Generalities

The Bcl-2 family shows diverse family protein members that originates from their first identified member, Bcl-2, in 1984. The Bcl-2 was discovered by the observation of t(14;18) chromosomal translocation in follicular lymphoma to evaluate the pathogenesis of B-cell neoplasms carrying the translocation (Erikson et al., 1984; Tsujimoto et al., 1984). Bcl-2 gene has been shown to promote hematopoietic cell survival and to cooperate with c-myc to immortalize pre-B cells (Vaux et al. 1988). If the BCL-2 protein is overproduced, the growth of Epstein-Barr virus-immortalized B cells is enhanced (Tsujimoto Y, 1989).

Bcl-2 is localized to the inner mitochondrial membrane that blocks programmed cell death (Hockenbery et al., 1990). Bcl-2 also localizes to the nuclear envelope, and endoplasmic reticulum (Krajewski et al., 1993). Overexpression of Bcl-2 in lymphoid tissues of Bcl-2-immunoglobulin transgenic mice develops a polyclonal expansion of small resting B cells. Thus the transgenic mice that overexpress Bcl-2 display extended survival of resting B cells (Nuñez et al., 1990). Bcl-2 also confers an antidote to apoptosis that aids malignant transformation (Hockenbery et al. 1991). Thus, Bcl-2 functions to inhibit apoptosis in a variety of in vitro and in vivo experiments, suggesting interference with a central mechanism of apoptosis (Hockenbery D.M., 1992).

Human Bcl-2 is a 26 kDa integral membrane oncoprotein consisting of five domains. The domains consist of four Bcl-2 homology (BH) domains named BH1, BH2, BH3 and BH4, and one transmembrane domain (Brunelle and Letai, 2009).

Apoptosis is a regulated process that is designed to eliminate cells with damage, superfluousness, and senescence (Glucksman A., 1951, Kerr et al., 1972, Wyllie et al., 1980). To date, Bcl-2 family consists of 25 pro- and anti-apoptotic members, and is grouped into three classes, according to their pro- and anti-apoptotic effects and the presence of Bcl-2 homology (BH) domains. The first class of the Bcl-2 family proteins such as Bcl-2, Bcl-2 related gene long isoform (Bcl-xL), Bcl-w, myeloid cell leukemia-1 (Mcl-1), and Bcl-2 related gene A1 (A1) inhibit apoptosis. These anti-apoptotic proteins have similar 3D structures and four BH domains. The BH domains that are possessed by the anti-apoptotic Bcl-2 family proteins (Brunelle and Letai, 2009) are crucial for the function of apoptotic control. The second class of the Bcl-2 family proteins are divided into multi-domain ‘effectors’ such as Bcl-2-associated-x protein (Bax), Bcl-2 homologueous agonist killer (Bak) and Bcl-2 related ovarian killer (Bok). They are pro-apoptosis proteins that contain up to 3 BH domains (Letai, 2008; Skommer et al., 2007; Youle and Strasser, 2008). If these effector proteins are once activated, Bak and Bak promote apoptosis by enabling pore formation within the mitochondrial outer membrane (Lindsten T., 2000).

The third class of the Bcl-2 family proteins are divided into the BH3-only ‘facilitators’ such as BH3 interacting-domain death agonist (Bid), Bcl-2-associated death promotorBad, Bcl-2 interacting mediator of cell death (Bim), Bcl-2-interacting killer (Bik), NOXA and p53-upregulated modulator of apoptosis (PUMA). The BH3-only group members inhibit the function of the anti-apoptotic members or promote the function of the multi-domain pro-apoptotic members (Letai, 2008; Skommer et al., 2007; Youle and Strasser, 2008). BH3-only protein signaling is essential for the initiation of the mitochondrial apoptotic pathway, but MOMP requires the presence of either Bak or Bak (Weyhenmeyer et al., 2012). Thus, regulation of apoptosis is recognized as an important function of Bcl-2 family proteins.
2.2 Bcl-2 and Endothelial Cell Survival

In 1994, Kondo et al. first showed a role for Bcl-2 in endothelial cell survival. In the study, they showed that apoptosis of murine aortic endothelial (MAE) cells was induced by deprivation of basic fibroblast growth factor (bFGF) but required new RNA and protein synthesis. Furthermore, enforced expression of Bcl-2 gene in MAE cells using gene transfer techniques decreased apoptosis induced by the deprivation of bFGF. Thus, Bcl-2 proteins protect endothelial cells against death induced by the removal of growth factors such as bFGF (Kondo et al., 1994).

In endothelial cells, Bcl-2 has an important correlation with vascular endothelial growth factor (VEGF). VEGF is a survival factor for endothelial cells and a key positive regulator of normal and pathological angiogenesis (Ferrara and Davis-Smyth, 1997), and Bcl-2 is one of the novel target genes of VEGF (Gerber et al., 1998). Overexpression of Bcl-2 by means of transient biolistic transfection experiments of primary human umbilical vein endothelial cells prevents from apoptotic cell death in the absence of VEGF (Gerber HP, 1998). Thus, Bcl-2 is sufficient to inhibit endothelial cell apoptosis.

On the other hand, Nör et al showed another role for Bcl-2 in endothelial cell survival. In the analysis, they demonstrated that implantation of Bcl-2-incorporated polylactic acid sponges into SCID mice exhibited an increase in the number of microvessels and a decrease in the number of apoptotic cells (Nör et al., 1999). The induction of Bcl-2 expression inhibits endothelial cell apoptosis mediated by thrombospondin-1, which inhibits angiogenesis in association with increased expression of Bax (Nör et al., 2000).

2.3 Bcl-2 and Angiogenesis

Angiogenesis is the growth of new blood vessels from pre-existing vasculature. It is an essential feature of pathological and physiological processes such as granulation tissue development, tissue repair and wound healing (Folkman, 1972; DeLisser et al., 1997). On the other hand, the effect of Bcl-2 proteins on angiogenic events is recently becoming more recognized as another major function of this molecule. For example, in tumor angiogenesis, a constitutive upregulation of Bcl-2, the first class of the Bcl-2 family proteins, in vascular endothelial cells is sufficient to enhance angiogenesis (Nör et al., 2001). The up-regulation of Bcl-2 in microvascular endothelial cells that constitute tumor microvessels accelerates tumor growth (Nör et al., 2001). In tumor cell lines, Bcl-2 expression is also an important factor of angiogenic potential. For example, xenograft tumors derived from Bcl-2-overexpressing prostate carcinoma cell lines that express high levels of VEGF display an increased angiogenic potential and grow more aggressively than tumors derived from control cell lines (Fernandez et al., 2001). Thus, Bcl-2 is a novel factor for tumor progression not only by the inhibition of apoptosis, but also by the induction of angiogenesis.

3 LCM-based Tumor Angiogenesis Research related to Bcl-2 Expression

LCM was first introduced as a system that is able to retrieve defined cell population from human tissue samples and developed during the mid-1990s by Dr. Emmert-Buck and col-
leagues at the National Institutes of Health (NIH), Bethesda, MD, USA (Emmert-Buck et al., 1996). Nowadays a variety of LCM apparatus are available such as the PixCell system (Arcturus, MDS Analytical Technology, California), Zeiss’s PALM system (a subsidiary of Carl Zeiss Microlmaging, Jana, Germany), Leica LMD system (Mannheim, Germany), and mmi CellCut Plus system (Molecular Machines & Industries (MMI), Switzerland). Their major differences relate to how they collect dissected cells.

Three types of LCM systems are currently available. The first type is the infrared (IR)-laser based LCM system such as the PixCell II and AutoPix. The IR laser-based LCM damages the tissue very little and is good for small targets, whereas it is not suitable for dissection of thicker samples, as compared with the ultraviolet (UV)-laser based LCM system mentioned below. The PixCell system was originated in a Cooperative Research and Development Agreement between NIH, the National Cancer Institute, and the National Institute for the Child and Human Development, and was manufactured/marketed by Arcturus.

The second type is the UV-laser based LCM system such as the Zeiss PALM, Leica LMD, and mmi CellCut. The UV-laser based LCM is suitable for clusters of cells and big areas of target tissues and possible to dissect thick sections such as those at 30 µm (Yamanaka et al., 2012). The Zeiss PALM system (Carl Zeiss, City, Germany) uses a pulsed UV-A laser to collect samples by photonic pressure termed laser pressure catapulting. The Leica LMD system (Leica, Mannheim, Germany) uses a UV laser to cut and then dissected cells fall into a collecting tube by gravity. The mmi CellCut Plus system uses a solid-state UV laser and manufactured/marketed by MMI, which was founded in 1998 by Prof. Stefan Seeger from University of Zurich, Switzerland.

The third type is the combined IR-UV laser system such as the Arcturus Veritas, and Arcturus XT (Arcturus). These systems use IR laser capture microdissection and UV laser cutting in one single instrument. A solid-state IR laser delivers a capture technique, which preserves biomolecular integrity and is ideal for single cells or a small number of cells. The solid-state UV laser delivers unprecedented speed and precision, suited for micro dissecting dense tissue structures and for rapidly capturing large numbers of cells.

4 Immune-LCM Analysis

To date, formaldehyde as a 10% neutral buffered formalin is most widely used as a fixative for various human tissues. The sections from formalin fixed samples normally show better preservation of tissue architecture, as compared with cryosections from frozen samples. As with DNA, formaldehyde reacts with RNA forming an N-methylol (N-CH2OH) followed by an electrophilic attack to form a methylene bridge between amino groups. Adenine is the most susceptible nucleotide to electrophilic attack and it is likely that the adenines within the mRNA sequence and the poly(A) tail of mRNA will be modified in the formaldehyde-fixated paraffin embedded sections to varying degrees. It is normally considered that RNA isolated from formaldehyde-fixated, paraffin embedded sections are less suitable for reverse transcription (cDNA synthesis), as compared to RNA isolated frozen tissue sections (Srinivasan et al., 2002). Thus, cryo-tissue sections are commonly used for gene expression analysis using LCM (Curino et al., 2004; Gandini et al., 2012) and immune-LCM. However a recent report has suggested that it is possible to perform gene expression analysis from approximately one-year-
old formaldehyde-fixated paraffin embedded tissue sections from human crista ampullaris (Pagedar et al., 2006).

We have further developed a methodology of immune-LCM of formaldehyde fixed, paraffin embedded HNSCC (head and neck squamous cell carcinoma) specimens that were immunostained for coagulation factor VIII (a marker of human endothelial cells), using the Leica AS LMD, which utilizes a pulsed 337 nm UV laser on an upright microscope (kaneko et al., 2011). The laser beam can be moved with a software-controlled mirror system, which allows us to select target cells and tissues: following selection of a target area on a monitor by a freehand drawing tool, the computer-controlled mirror moves the laser beam along the pre-selected path and the laser beam cuts the target tissue. The dissected tissue sections fall into a cap of PCR tube by their gravity. The collection by gravity is very fast and it is easy to transfer the dissected tissue sections to reaction buffer. The thickness and width of the cutting line can be controlled for each tissue. Thus, this method allows us to collect good quality RNA of factor VIII-positive endothelial cells retrieved from the tumor mass.

4.1 Immune-LCM Analysis for Bcl-2 Expression

By immune-LCM using formaldehyde-fixated paraffin embedded samples, we have reported that the crosstalk between dermal micro-vascular endothelial cells and tumor cells plays an important role in oral squamous cell carcinoma (OSCC) growth and angiogenesis, and that Bcl-2 is a key regulator of this crosstalk. Thus, a novel role for Bcl-2 in endothelial cells as a pro-angiogenic signaling molecule has been presented (kaneko et al., 2007). Our immune-LCM analysis has also demonstrated that oral administration of a small-molecule inhibitor of VEGF receptors (PTK/ZK) is anti-angiogenic in early stage head and neck tumors, which are accompanied by quantifiable inhibition of the VEGF-Bcl-2-CXCL8 signaling axis (Miyazawa et al., 2008). In another immune-LCM analysis, we investigated signaling pathways involved in the regulation of Bcl-2 in lymphatic endothelial cells, and the impact of endothelial cell Bcl-2 expression in primary tumors on six cases of lymph node metastasis. In the analysis, we have further reported that Bcl-2 in endothelial cells contributes to lymph node metastasis in patients with OSCC (Tarquino et al., 2012). Thus, this method using formaldehyde-fixated paraffin embedded samples may be better suited for the analysis of relatively rare cell types within a tissue, and may improve our ability to perform differential diagnosis of pathologies as compared to conventional LCM (kaneko et al., 2009).

Recently, another immune-LCM analysis of endothelial cells in angioimmunoblastic T-cell lymphoma has been reported (Ratajczak et al., 2012). In the analysis, immune-LCM was performed for 7 µm-thick lymph node frozen sections following immunofluorescent staining of CD34- or CD105-expressing endothelial cells, and showed a significant relationship between Bcl-2 and VEGFA mRNA levels in CD34-expressing endothelial cells.

4.2 Immune-LCM from Formaldehyde-fixated, Paraffin-embedded Oral Cancer Tissues

This protocol is modified from our previous report (kaneko et al., 2011, 2013). Protocols of measurement of RNA concentration and real-time PCR experiments are newly added, and some reagents (cDNA reverse transcription reagents) are changed. Disposable gloves should always be worn as skin often contains bacteria and molds that can be a source of contaminating RNA and RNases, The use of sterile, disposable plastic ware and filtered pipette tips is
recommended for RNA work to prevent cross-contamination with RNases from shared equipment.

4.2.1 Tissue Fixation, Paraffin Embedding

1. Fix tissues with 10% neutral buffered formaldehyde for 8 – 16 hours at 4 °C.
2. Dehydrated for 30 minutes in 70% ethanol, for 1 hour in 90% ethanol, and for 30 minutes in 95% ethanol at 4 °C.
3. Dehydrated 3 times for 1 hour in 100% ethanol at room temperature.
4. Immersed 2 times in xylene for 1 hour at room temperature.
5. Immersed 4 times in paraffin for 30 minutes at 58 °C.
6. The specimen is embedded in paraffin and blocked.

4.2.2 Paraffin block storage

1. We recommend that the paraffin blocks be stored at 4 °C, but the paraffin blocks can be stored at room temperature until processed.
2. The blocks can be kept for 12 months, but we recommend that the blocks should be sectioned as soon as possible.

4.2.3 Sectioning and mounting

1. Cut sections on a microtome with a new sterile disposable blade (8 – 10 µm thick).
2. Float paraffin ribbons on 43 °C nuclease-free water (diethylpyrocarbonate (DEPC)-treated water).
3. Mount the section on poly-L-lysine coated glass foiled polyethylene naphthalate (PEN) slides for LCM (Leica)
4. Dry slides in a 35 °C incubator for 6 hours.

4.2.4 Slide storage

1. We recommend that the slides be stored at 4 °C and LCM should be performed as soon as possible. Slides should be used within a week after preparation.

4.2.5 Staining: nuclear staining by hematoxylin

1. Deparaffinize the slides 3 times with 2 minutes xylene washes at room temperature.
2. 100% ethanol washes 3 times for 20 seconds at 4 °C.
3. 90% ethanol wash for 30 seconds at 4 °C.
4. 70% ethanol wash for 1 minute at 4 °C.
5. DEPC-treated water wash for 30 seconds at 4 °C.
6. Gill No.3’ hematoxylin (Sigma-Aldrich, Deisenhofen, Germany) for 5 – 10 seconds at room temperature.
7. DEPC-treated water wash for 30 seconds at 4 °C.
8. Air dry the slide for 1 – 3 hours at 4 °C.

4.2.6 Staining: immunostaining

Avidine-biotin-peroxidase complex (ABC) method is employed. 2 ~ 4 slides per session is easy to manageable.

1. Deparaffinize the slides twice with 3 minutes xylene washes at room temperature.
2. 100% ethanol washes 3 times for 30 seconds at 4 °C.
3. 90% ethanol wash for 30 seconds at 4 °C.
4. 70% ethanol wash for 1 minute at 4 °C.
5. RNase-free phosphate buffered saline (PBS) washes 3 times for 30 seconds at 4 °C.
6. Pre-treat these sections with 0.125% trypsin for 5 ~ 30 minutes at room temperature. To avoid RNA degradation (Vrtacnik et al., 2014), pre-treatment of trypsin should be performed at room temperature.
7. RNase-free PBS washes 3 times for 30 seconds at 4 °C.
8. Endogenous peroxidase activity blockage with 0.3% hydrogen peroxide in methanol for 3 minutes at 4 °C.
9. RNase-free PBS washes 3 times for 30 seconds at 4 °C.
10. Incubation with a primary antibody for 16 h at 4 °C, or for 30 minutes at room temperature.
11. RNase-free PBS washes 3 times for 30 seconds at 4 °C.
12. Incubation with biotinylated secondary antibody (Vector laboratories, Burlingame, CA; diluted 1:500) for 1 hour at 4 °C.
13. RNase-free PBS washes 3 times for 30 seconds at 4 °C.
14. Incubation with avidine-biotine-peroxidase complex (Elite ABC kit; Vector) for 1 hour at 4 °C.
15. RNase-free PBS washes 3 times for 30 seconds at 4 °C.
16. Development in diaminobenzidine-H2O2 solution (DAB substrate kit; Vector) for 3 minutes at room temperature.
17. RNase-free PBS washes 3 times for 30 seconds at 4 °C.
18. Air dry the stained slides for 1 – 3 hours at 4 °C.
19. Store at ~70 °C ~ 4 °C until ready to perform LCM.

4.2.7 LCM (Leica AS LMD system) and total RNA extraction by TRIZOL® Reagent (Life Technologies™ (Invitrogen™), Carlsbad, CA)

1. Dissect Factor VIII-positive endothelial cells from tumor mass (Figure 1A & B) or control normal mucosa (Figure 1C & D) by using a LCM system.
2. Collect dissected endothelial cells (approximately 400 cells) into individual tubes filled with 20 µl TRIZOL® Reagent. Tubes should be immediately placed on ice.

3. Close the tubes securely. Centrifuge the samples at 14,000 rpm for 10 seconds at 4 °C.

4. Add TRIZOL® Reagent to sufficient to 30 – 100 µl.

5. Incubate the samples for 5 minutes at room temperature.

6. Add chloroform in the same amount as TRIZOL® Reagent used.

7. Close sample tubes securely. Shake tubes briefly for 30 – 60 seconds and incubate them for 5 minutes on ice.

8. Centrifuge the samples at 14,000 rpm for 15 minutes at 4 °C.

9. Transfer the aqueous phase to a fresh tube. (Following centrifugation, the mixture separates into three layers; a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA is contained exclusively in the aqueous phase.)

10. Add isopropyl alcohol (RNA precipitant) to the tube in the same amount as the aqueous phase transferred. Incubate the samples at 4 °C for 10 minutes. Centrifuge at 13,000 rpm.
for 15 minutes at 4 °C. RNA will be precipitate under the tube. The RNA precipitate forms a white small dotted pellet on the side and bottom of the tube. The RNA precipitate is sometimes invisible after centrifugation,

11. Discard the supernatant gently.
12. Add 70 – 75% ethanol in DEPC-treated water to the RNA pellet and mix the sample by vortex.
13. Centrifuge at 6000 – 7500 rpm for 5 minutes at 4 °C.
14. Discard the supernatant gently.
15. Add 70 – 75% ethanol in DEPC-treated water to the RNA pellet,
16. The RNA pellet can be stored at -20 °C.

4.2.8 RNA clean up by RNeasy Mini Kit (Qiagen, Frederick, ML)

All processes of RNA clean up by RNeasy Mini Kit can be performed at room temperature.

1. Add 10 µl β-Mercaptoethanol per 1 ml Buffer RLT.
2. Adjust the sample to a volume of 100 µl with RNase-free water. Add 350 µl Buffer RLT, and mix briefly.
3. Add 250 µl of 100% ethanol to the diluted RNA, and mix by pipetting without centrifuge.
4. Transfer the sample (total 700 µl) to an RNeasy Mini spin column placed in a 2 ml supplied collection tube. Centrifuge for 15 seconds at 8000 x g. Discard the flow-through.
5. Reuse the collection tube.
6. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 seconds at 10000 rpm to wash the spin column membrane and discard the flow-through.
7. Reuse the collection tube in step 4.
8. Add 10 µl DNase I stock solution to 70 µl Buffer RDD that is supplied with the RNase-Free DNase Set. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
9. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place at room temperature for 15 minutes.
10. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 seconds at 10,000 rpm. Discard the flow-through.
11. Add 500 µl Buffer RPE to the RNeasy spin column, and centrifuge for 15 seconds at 10,000 rpm and discard the flow-through.
12. Reuse the collection tube.
13. Add 500 µl Buffer RPE to the RNeasy spin column, and centrifuge for 2 minutes at 10,000 rpm.
14. Place the RNeasy spin column in a new 2 ml collection tube, and discard the old collection tube with the flow-through, and centrifuge at 13,000 rpm for 1 minute.
15. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 10 – 20 µl RNase-free water directly to the spin column membrane, and centrifuge for 1 minute at 10,000 rpm to elute the RNA.

16. Store at −70 °C until ready to perform single-stranded cDNA synthesis.

4.2.9 Measurement of RNA concentration and absorbance value by a photometer
1. Add 1 ~ 2 µl of RNA into DEPC-treated water to sufficient to 100 µl.
2. Prepare blank solution (100 µl DEPC-treated water).
3. Fill a disposable rectangular plastic cuvette (Eppendorf UVette) with blank solution.
4. Insert the filled cuvette with the blank solution into a cuvette shaft of photometer (Bio-Photometer plus, Eppendorf). Measure the blank solution.
5. Insert the filled cuvette with sample solution into a cuvette shaft of photometer. Measure the concentration result and absorbance value of the sample solution.

4.2.10 Single-stranded cDNA synthesis
1. Add 1 µg of total RNA (up to 10 µl) into 10 µl 2X master mix (High Capacity cDNA Reverse Transcription Kits, Life Technologies™ (Applied Biosystems®)).
2. Add DEPC-treated water to sufficient to 20 µl.
3. Seal PCR tubes securely, centrifuge briefly and centrifuge the tubes to eliminate air bubbles for 10 seconds at 4 °C.
4. Place the tubes on ice until ready to load a thermal cycler (TaKaRa PCR Thermal Cycler Dice®, Takara BIO INC, Siga, Japan).
5. Program the thermal cycler (set the cycler program at the reaction volume of 20 µl).
6. cDNA synthesis and pre-denaturation: perform 1 cycle at: 25 °C for 10 minutes, 37 °C for 120 minutes 85 °C for 5 minutes.
7. Start the reverse transcription of the cycler run.
8. Add DEPC-treated water to sufficient to 100 µl.
9. Store at −70 °C until ready to perform real-time PCR (or other PCR applications).

4.2.11 Real-time PCR experiments (StepOne™, Applied Biosystems®)
1. Add 1 µl of TaqMan Gene Expression Assay (Bcl-2, Hs00608023_m1 or 18S, Hs99999901_s1) and 1 µl of cDNA template into 10 µl 2X TaqMan Gene Expression Master Mix (Applied Biosystems®).
2. Add 8 µl of DEPC-treated water to sufficient to 20 µl.
3. Seal a 48-well clear optical reaction plate securely, centrifuge briefly and centrifuge the tubes to eliminate air bubbles for 10 seconds at 4 °C.
4. Program the thermal cycler (set the cycler program at the reaction volume of 20 µl)
5. PCR: perform 1 cycle at: 50 °C for 2 minutes and 95 °C for 10 minutes, and perform 35 ~ 40 cycles at: 95 °C for 15 seconds and 60 °C for 1 minutes.

Figure 2 shows a graph of Statistical comparison of two experimental groups (endothelial cells retrieved from control normal mucosa vs. endothelial cells retrieved from inside the tumor mass from the same tissue arrays. Data presented from real-time PCR experiments reflect the expression level of Bcl-2 normalized by 18S.

![Figure 2](image)

Figure 2: Bcl-2 mRNA expression in endothelial cells retrieved from tumor mass or control normal mucosa. Data presented from real-time PCR experiments reflect the expression level of Bcl-2 normalized by 18S.

5  Conclusion

Tissue based LCM is a powerful technique that combines morphology, histopathology and molecular biological analysis. The ability of LCM to retrieve specific populations of interested cells, combined with the analysis of gene sequencing and gene expression in these sub-population of cells, has made LCM a critical strategy in tumor angiogenesis research related to Bcl-2 expression.

References


