DESIGN OF A QPCR EXPRESSION ARRAY OF APOPTOSIS REGULATORS

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INTRODUCCTION

Apoptosis is a process of programmed cell death and can be induced by a number of stimuli, including binding of ligands to membrane receptors, deprivation of growth factors and DNA damage. This cell death process is characterised by chromatin condensation and contraction of the nucleus and cytoplasm, followed by fragmentation of the cell in apoptotic bodies, which are quickly engulfed by phagocytic cells and digested in lysosomes. There are two pathways of apoptosis: extrinsic and intrinsic. The difference between both is the participation of different members of the Bcl-2 protein family. Defects in the control of apoptosis contribute to many human diseases, particularly cancer. It is well known that glioblastoma (GBM) cells are highly resistant to apoptosis induced by chemotherapy and radiotherapy. On the other hand, testicular germ cell tumor cells (TGCTs) are highly sensitive to apoptosis induced by cisplatin.

HYPOTHESIS

Because of the high number of apoptosis regulators it would be very helpful to have a home-made assay to analyze most of these regulators in a simple and quantitative assay. Thus, we have optimized a qPCR assay in 96-well plates to quantitate the expression levels of 17 genes involved in the regulation of apoptosis, including members of the Bcl-2 family and the IAP family.

MATERIALS AND METHODS

•Cell culture. Gliomablastoma cells from surgical specimens were cultured in DMEM/F12 medium supplemented with B27, EGF and βFGF whitout serum. NTERA2 cells (TGCTs) were maintained in DMEM supplemented with 10% fetal calf serum.
•Substrate. Collagen-coated plates or collagen matrices were used to culture GBM cells. Collagen I was used at a concentration of 1.8 mg/ml.
•Primer design. Oligonucleotides were designed with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and verified by searching at BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
•qPCR. The real-time PCR assay was performed by using an ABI PRISM 7000 equipment (Applied Biosystems) with SYBR Green PCR Master Mix.

RESULTS

1. Design of qPCR array

Table 1. Selection of Primers for the array

Anti-apoptotic genes	Primer forward	Primer reverse	Product length (bp)
Bcl-2	CATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGGTACTCAGTCA	82
Bcl-X	CTCCCTCAGCGCTTGCTTTA	CGCACAGCAGCAGTTTGG	65
Mcl-1	CAAGGATGGGTTTGTGGAGT	TAGATATGCCAAACCAGCTC	112
Pro-apoptotic genes			
Bax subgroup gene			
Bax	TGGAGCTGCAGAGGATGATTG	CCAGTTGAAGTTGCCGTCAGA	100
Bak	ACGGCAGAGAATGCCTATGA	AGAACCACACCCAGAACCAC	289
BH3 subgroup genes			
BNIP3L	GTGGAAATGCACACCAGCAG	CTTGGGTGGAATGTTTTCGG	140
Bid	GATGAGCTGCAGACTGATGGC	GATGCTACGGTCCATGTGTC	140
Bad	AGCCATCATGGAGGAGCT	ATGTGGAGCGAAGGTCACTG	346
Bim	CACAAACCCCAAGTCCTCCTT	TTCAGCCTGCCTCATGGAA	73
Puma	ACGACCTCAACGCACAGTACG	TGGGTAAGGGCAGGAGTCC	110
Noxa	AGCTGCGTTTCACCAGGG	TCCAGCTACTTGCACTTGTTCCT	101
IAP family members			
IAP1	TGAGCATGCAGACACATGC	TGACGGATGAACTCCTGTCC	249
IAP2	CAGAATTGGCAAGAGCTGG	CACTTGCAAGCTGCTCAGG	273
XIAP	TGGCAATATGGAGACTCAGC	TGCACTTGGTCACCAATAC	380
Survivin	GCCAGATGACGACCCCATAG	CGCACTTTCTCCGCAGTTTC	192
Regulating genes			
SMAC	TCTGAAGAGTTGGCTGTCGC	CTGTGCAATAGGAACCGCAC	175
APAF1	CCTGTTGTCTCTTCTTCCAGTGT	CTGAAACCCAATGCACTCCC	253

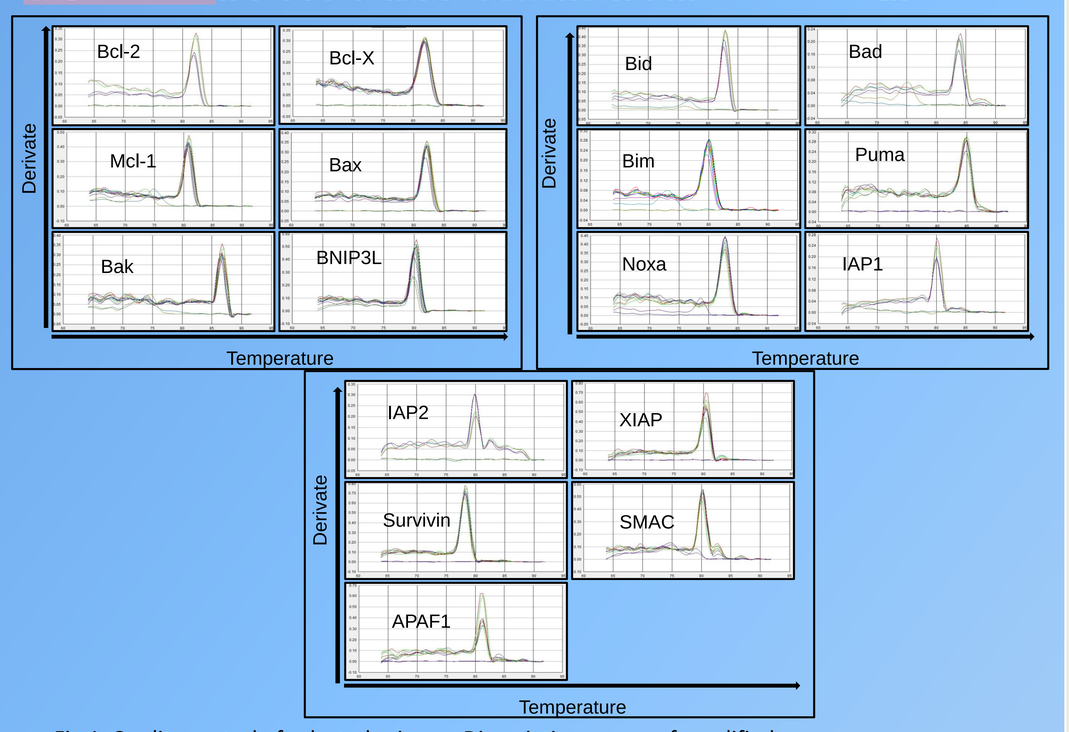

Fig.1. Quality control of selected primers. Dissociation curves of amplified genes

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A													B-actina
B													G6PD
C													Bcl-2
D													Bcl-X
E													Mcl-1
F													Bax
G													Bak
H													
	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	

Sample 1 Sample 2 Sample 3 Sample 4

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12	
A													B-actina
B													G6PD
C													BNIP3L
D													Bid
E													Bad
F													Bim
G													Puma
H													Noxa
	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	

Sample 1 Sample 2 Sample 3 Sample 4

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12	
A													B-actina
B													G6PD
C													IAP1
D													IAP2
E													XIAP
F													Survivin
G													SMAC
H													APAF1
	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35	P36	

Sample 1 Sample 2 Sample 3 Sample 4

Fig.2. Template of qPCR array of apoptosis regulators

2. Cellular models: GBM cells treated with sunitinib under different culture conditions (Fig. 3, 4) and testicular germ cell tumor (TGCT) cells treated with cisplatin (Fig. 5).

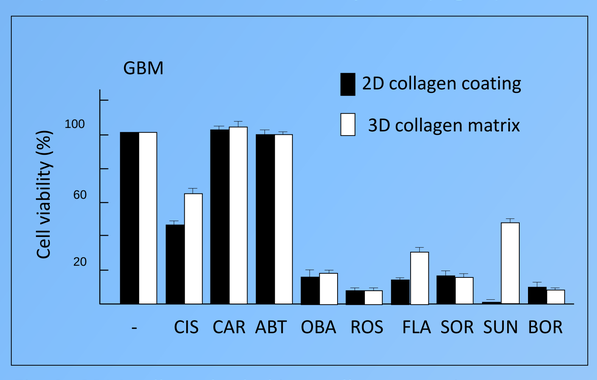

Fig. 3. GBM cells embeded in a collagen matrix are protected against sunitinib-induced apoptois

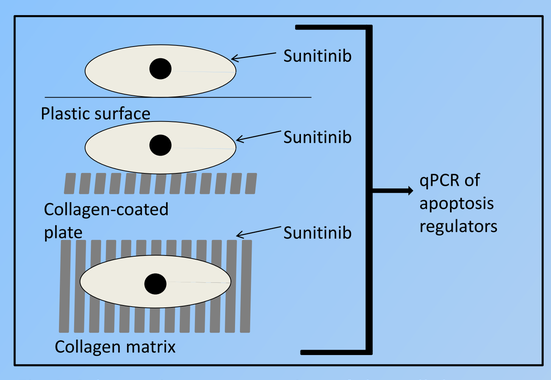

Fig.4. Schematic representation of the cellular model used to try the apoptosis qPCR array.

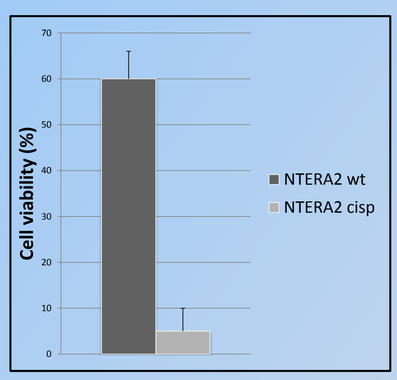

Fig. 5. Cell viability of TGCT cells treated with cisplatin, which shows the high sensitivity to this genotoxic agent

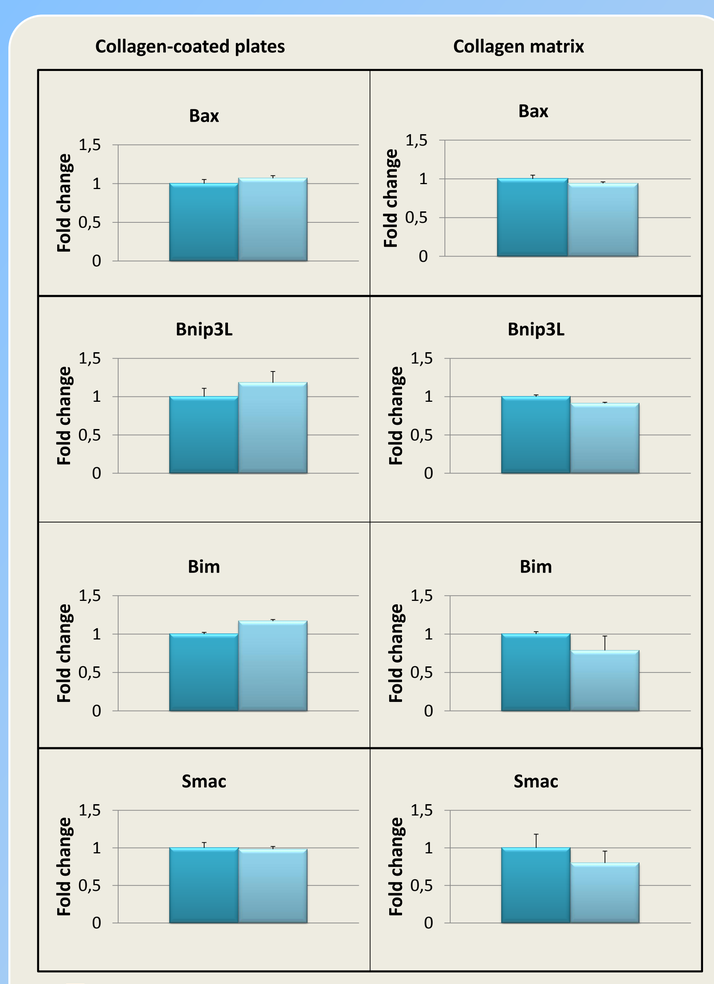

Fig.6. Expression of apoptotic genes in **GBM** cells cultured on collagen-coated plates or within collagen matrix following treatment with sunitinib. The figure only shows those genes with significant changes in expression, Bim and Smac, between both culture conditions. Bax and Bnip3L are included for comparison. Expression levels are normalized to b-actin

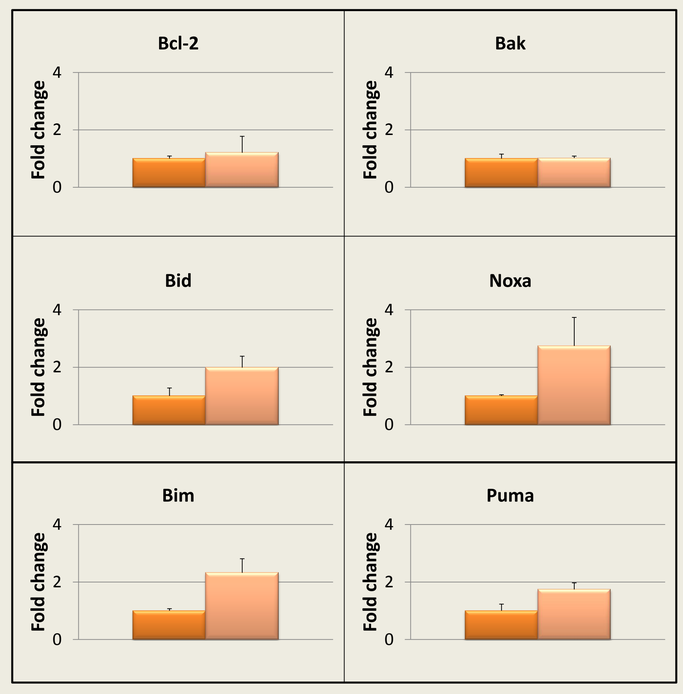

Fig.7. Expression of apoptotic genes in **NTERA2** cells treated with cisplatin. A significant increase in Noxa, Bid, Bim and Puma levels is observed in cells treated with cisplatin. Bcl-2 and Bak are included for comparison. Expression levels are normalized to b-actin

CONCLUSIONS

- We designed a qPCR array to study the expression of 17 genes involved in the regulation of apoptosis.
- We have optimized primers and PCR conditions for optimal quantitation of apoptosis regulators
- We have validated the qPCR array by using two models of tumor cells, primary GBM cells and testicular germ cell tumor cells (TGCTs).
- GBM cells are sunitinib-resistant when cultured in a 3D collagen matrix and we observe a reduction in the expression of proapoptotic genes Bim and Smac.
- TGCT cells are highly sensitive to cisplatin and we detect an increase in proapoptotic genes Noxa, Bid, Bim and Puma.

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