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Gene Expression Profile in Liver Transplantation and the Influence of Gene Dysregulation Occurring in Deceased Donor Grafts

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Abstract: *Background*: Brain dead patients are the main source of organs for transplants. Brain death causes changes in peripheral organs. We define modifications of gene expression in specific pathways occurring in donor livers and their influence on gene expression profile of livers after transplant.

Methods: We compared gene expression profile of both deceased donor livers and transplanted livers to gene expression data of liver tissue, retrieved from Array Express database, used as control. All expression data were obtained by microarray analysis.

Results: The expression of about 33,000 genes has been compared in liver samples from three groups: deceased donor livers, transplanted livers two hours after reperfusion, and control livers. We found that about 900 genes are dysregulated in deceased donor versus control livers. Up-regulated genes are mainly involved in apoptosis, immune response and inflammation. Down-regulated genes are mostly involved in metabolism and electron transport. We also re-evaluated a group of genes that in a previous study were found dysregulated in transplanted livers when compared to donor livers. Most of these genes, but not all, were dysregulated also when compared to control livers. Moreover 317 additional genes, dysregulated after liver transplant, were identified in this study; they were undetectable in the previous study because they had the same dysregulation both in donor and in transplanted livers.

Conclusions: Understanding molecular mechanisms that in the donor compromise graft function is crucial in order to discriminate between basal graft damages and ischemia-reperfusion injuries and therefore to identify therapeutic targets aiming to improve liver transplantation performances.

Keywords: Liver transplantation, brain death, gene expression profiling, ischemia/reperfusion injury.

INTRODUCTION

Organs from brain dead donors are the main source for solid organ transplantation. Brain death (BD) is a complex physiological event, defined as an irreversible injury of cerebrum, cerebellum and brain stem and is associated with severe hemodynamic changes, coagulopathies, pulmonary changes, hypothermia and electrolyte imbalances. Hemodynamic instability associated with brain death can contribute to deterioration of peripheral organs. These changes may predispose the graft to increasing ischemia reperfusion injury (IRI) damages during the transplant process, accelerating organ rejection after transplantation [1]. The hemodynamic instability is well recognized as 'autonomic storm', an initial period of excessive parasympathetic activity, which is immediately followed by a sympathetic activation with high plasma levels of catecholamines, extreme arterial hypertension and tachycardia. During these phases, potential donor grafts undergo a transient period of ischemia [2]. Shock and oxidative injury during the intensive care treatment of the potential donor, followed by brain death, should be regarded as a major risk factor affecting organ viability, post-transplant function and graft survival in organ transplantation, in addition to unavoidable IRI during transplantation procedures. Donor's brain death might cause and promote organ injury altering the immunological and inflammatory status of the graft, increasing both the sensitivity of the organs towards preservation injury and acute rejection following transplantation. Despite this correlation has been experimentally shown, no clinical trials support so far this hypothesis [2].

In a previous study [3] we have compared gene expression levels in transplanted livers, soon after reperfusion, versus basal gene expression levels, before liver retrieval from the donor. About 800 genes were found dysregulated after transplantation, but we have not analyzed the potential effects of brain death on the gene expression variations.

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A very recent study [4] compares gene expression patterns of transplanted livers from living donors with transplanted livers from deceased donors, defining molecular signature of both. The authors focus their attention to the pathways, which show dysregulation in the transplant process both in livers from living donors and from deceased donors; however they do not analyze the basal differences between the grafts from the two groups of donors.

The aim of the present study was to systematically define alterations of gene expression and impairment of specific pathways induced in deceased donor livers when compared to control livers. Based on these alterations an accurate analysis of genes dysregulated in transplanted livers versus control livers was carried out, in order to discriminate between transcriptional changes, due to ischemia reperfusion injuries, and variations possibly caused by brain death and other donor conditions.

We have compared gene expression profile of both deceased donor livers (DL) and transplanted livers (TL) to gene expression data of liver tissue, retrieved from Array Express database, used as control (CL) [3, 5]. This control was a set of livers from sudden death individuals without previous agonal state. Literature data demonstrate that the integrity of mRNA is scarcely affected by sudden death without agonal state. In contrast agonal state preceding death has a substantial effect on gene expression [6, 7]. A very large amount of research studies have been conducted on deceased tissues and also information included in all the databases reporting normal gene expression throughout the tissues [8] is derived from autoptic studies.

The expression of about 900 genes was found dysregulated in DL if compared to controls. This wide gene expression modification clearly affects gene regulation in TL.

The data reported in the present study give new insight to clarify the consequences of brain death and intensive care injuries on the human orthotopic liver transplantation by a molecular point of view, and help us to recognize new therapeutic targets useful to improve orthotopic liver transplantation (OLT) performance.

MATERIALS AND METHODOLOGY

Experimental Design

Gene expression profile of samples from DL and TL was compared to gene expression profile of samples from CL. The choice of sample size was performed by running p-values of expression data comparisons among groups through the PowerAtlas software [9, 10]. We selected a sample size of '5' per group to obtain an Expected Discovery Rate (EDR) > 40% (63%) and a True Positive Probability (TP) > 80% (98%) (Supplementary file **1**).

Then, five biological replicates per condition were analyzed. All expression data (DL, TL and CL) were obtained by microarray analysis, using Affymetrix gene chip HG-U133 Plus 2. Hybridization data were normalized and quantified using Robust Multiarray Analysis (RMA) software [11]. DL and TL samples were collected as previously described [3]. Briefly, 10 liver biopsies were analyzed: 5 biopsies, from the donors, collected before explantation (DL group); 5 biopsies from the transplant recipients (TL group), collected 2-3 hours after liver reperfusion. Donors were classified as 'standard' according to the criteria of the 'Italian National Transplantation Center'. Particularly, the donors' age ranged from 38 to 83 years, no hypotension, steatosis always less than 15%. Mean cold ischemia time was 8 hours (ranging from seven to ten hours), mean warm ischemia 45 min, mean hospitalization in intensive care unit was 5 days [3]. Expression data of livers, from five healthy individuals who suffered sudden death, were retrieved from Array Express database (SAMPLE ID: E-AFMX-11) [5, 12] and used as control samples. All individuals, 3 males and 2 females, suffered sudden death for reason other than their participation to the study and without any relation to the tissues used. Age was ranging from 27 to 29 and was unknown in 2 cases. Total RNAs, isolated from 200 mg of frozen tissues using the Trizol reagent, were of high and comparable quality as gauged by the ratio of 28S to 18S ribosomal RNAs estimated using the Agilent 2100 Bioanalyzer (range 1.4-1.6) [12].

In order to validate the use of deceased tissues as controls, we compared our results to a set of expression data from microarray analysis (same technology and experimental conditions) of six liver biopsies from living donors, available very recently from GEO repository [4, 13] (Supplementary file **2**).

RNA Extraction, Data Acquisition and Analysis

DL and TL samples were treated as previously described [3]. Array data have been deposited in GEO data base with accession number GSE14951 [14]. Expression data were pre-filtered to reduce noise and discard 'unreliable' genes using the Cross-Gene error model [14]. The analysis of functional clusters was performed on lists of differentially expressed genes for both Gene Ontology (GO) categories and biological pathways. GO functional class scoring was performed using the web-based GOTM software [15, 16] which visualizes differentially expressed genes in the GO context, considering as gene sets all the GO categories for biological processes, molecular functions and cellular components. The list of differentially expressed genes was compared to the complete list of genes spotted on Affymetrix HG-U133 Plus 2 chip, in order to identify GO categories of genes significantly ($p < 0.01$) more represented in the list of differentially expressed genes than in the reference gene set.

Real-Time Quantitative PCR

Expression values of 26 genes chosen among the most dysregulated genes were checked by RT-PCR. The same batch of total mRNA was used for both microarray and validation experiments in DL and TL. RNAs, from five liver tissue obtained during resection of benign focal lesions, were used as controls in RT-PCR experiments. Two biopsies came from liver resections of two females (24 and 26 aged) suffering by hepatocellular adenoma. Two biopsies came from liver resections of giant hepatic cystis from two male aged respectively 39 and 43. The last biopsy came from a liver of a male suffering by liver hemangioma. cDNA was synthesized Real Time PCR were performed as previously described [3]. PCR reactions were performed in triplicate. The primers (Primm Biotech Products and Services, Milan -

Table 1. Primer Pairs Used for Real-Time Quantitative PCR

Probe Set	Gene Name	Ensembl Gene ID	Left Primer	Right Primer
205364_at	ACOX2	ENSG00000168306	CGGAGTCTTCAGGACCACAC	GCAGGAAGCCATTGTCTGTT
231587_at	APOC3	ENSG00000110245	CAGCCCCGGGTACTCCTT	TTGGTGGCGTGCTTCATGTA
209186_at	ATP2A2	ENSG00000174437	TGAAACAGTTCATCCGCTACC	AATCAAAGCCTCGGGAAATC
228876_at	BAIAP2L2	ENSG00000128298	AGAACGTGCGGGAGATGAAG	CAGACACGAAGGCCTGCAT
212952_at	CALR	ENSG00000179218	CCTGAGTACAAGGGTGAGTGGAA	GCCCAGCACGCCAAAGT
204093_at	CCNH	ENST00000256897	CAGAAGTTGGAGCGATGTCA	GTCCATTCTTCCTCCTCATGTT
220046_s_at	CCNL1	ENSG00000163660	GAACTCCAGCCCTTTCAACC	TTGGTGATTTCTCTTCAGCTTTT
228766_at	CD36	ENSG00000135218	GCTGAGGACAACACAGTCTCTTTC	AGCCTCTGTTCCAACTGATAGTGA
213279_at	DHRS1	ENSG00000157379	TGGCCGTGGCATTGC	AGATGGCGGCCAGTGATG
1555612_s_at	G6PC	ENSG00000131482	TCCGTCAGTGTCATCCCCTACT	CCGAAGACTCCACATCTCTTACAA
210328_at	GNMT	ENSG00000124713	ATCATCGCAACTACGACCACAT	GTCCTTGGTCAAGTCACTCTTATAGTAGAT
215554_at	GPLD1	ENSG00000112293	GGGACCAGTGACTGCAACCT	GCCCTGGGTGTGGTTTTG
241945_at	HECTD1	ENSG00000092148	ACTAATGCCACGAACAACATGAAT	TGATGTAGTACCAGGTGTGGTCAA
201466_s_at	JUN	ENSG00000177606	AGAAAGTCATGAACCACGTTAACAGT	CCCCCGACGGTCTCTCTT
205222_at	LBP	ENSG00000129988	CCGACTGACCACCAAGTCCTT	GGCACTGATCCCTGGAGTTC
203675_at	NUCB2	ENSG00000070081	GAAAAGGCAAGAAGTAGGAAGGTT	GTCAGGATTCAGGTGGTTTAGG
206278_at	PTAFR	ENSG00000169403	CCTGCCACTTTGGATTGTCTACT	GCCACGTTGCACAGGAATTT
210479_s_at	RORA	ENSG00000069667	TCATTCTCCACCCAGCTGTTG	CTGTGCTTTGCCCCAGTGTA
222226_at	SAA3P	ENSG00000166787	GCCAGGCTACCAACAAATGG	GCAGATTGAAAAGGAAGCTCAGTAT
213874_at	SERPINA4	ENSG00000100665	TCAAAGCCCTGTGGGAGAAA	CGGACTGTTGTGTTCTCATCAAC
222705_s_at	SLC25A15	ENSG00000102743	CAGCCGCCGGTTCCTT	ACACTGTATTCTGGCTCTTGGCTAT
215223_s_at	SOD2	ENSG00000112096	TGGTGGTGGTCATATCAATCA	GCCGTCAGCTTCTCCTTAAA
217040_x_at	SOX15	ENSG00000129194	CAGCGGATTTTGCATTCGA	GCTTAAACCGGAGCCTTTGC
207306_at	TCF15	ENSG00000125878	CCAGAGGGTATGTGTTGAAAAGTCT	CCCTAGGCTGCTTGCAGAGA
201042_at	TGM2	ENSG00000198959	CTTTGACGTCTTTGCCCACAT	CGGTGCGGGCACAGA
239818_x_at	TRIB1	ENSG00000173334	GGGCGCTGTGCATCCA	AAGGCCTGATTTTGTCCTGGTA

Italy) used for amplification are listed in Table **1**. Primer pairs were designed using the Primer 3 software [17] in order to obtain amplicons ranging from 100 and 150 bp, and specifically designed to span introns or cross intron/exon boundaries. Data normalization was performed using *GAPDH* as housekeeping gene [18]. Experiments were performed twice, in triplicate. The amplification protocol was: 1 cycle of 10 min at 95 °C, 40 cycles of 95 °C for 15 sec, 58-60 °C for 20 sec, 72 °C for 20 sec, plus an extension at 72 °C for 3 min. The relative expression value was calculated with the formula 2^{DDct}

Statistics

Expression data from different groups were compared using the ANOVA test, with Benjamini and Hochberg false discovery rate as multiple testing corrections. Statistical significance was established at $p < 0.01$. Genes were considered differentially expressed with a fold change > 1.5 between two conditions. Gene sets were considered enriched with a $p < 0.01$ when compared to the reference gene list.

Liver biopsies were collected in different hospitals, including the “Liver Transplantation Center” of the “Cardarelli Hospital”. All biopsies were obtained with informed consent given according to protocols approved by the Institutional Ethics Committee of the “Antonio Cardarelli Hospital.”

RESULTS

The expression of about 33,000 genes, represented on the Affymetrix chip HG-U133 Plus 2, has been evaluated in liver samples from three groups: DL, TL 2 hours after reperfusion, and CL. The groups were compared each other for gene expression. Expression of about 900 genes was dysregulated in DL compared to control ones. Table **2** shows the 40 most up-regulated and the 40 most down-regulated genes. Table **3a** and Table **3b** report the distribution of the dys-

Table 2. The Most up- and Down-Regulated Genes in DL Group *vs*. CL Group

Up-Regulated Genes		Down-Regulated Genes	
Gene Name	**Fold Change**	**Gene Name**	**Fold Change**
SPINK1	95.29	LOC283130	0.0555
APOC3	9.516	MALAT-1	0.0612
RPS11	8.807	TF	0.084
SOD2	7.177	KCNN2	0.0927
PLEKHG5	6.343	ID2	0.106
LOC440836	6.052	H19	0.112
ATP2A2	5.483	CAT	0.116
APOA1	5.461	GNMT	0.117
TOMM40	5.194	G6PC	0.13
SAP30L	5.043	CD36	0.131
TMEFF2	5.042	RORA	0.135
APOC2	4.937	C8orf4	0.139
TMEM151	4.83	SLC6A1	0.145
RBP4	4.765	HECTD1	0.146
C9ORF44	4.72	KIAA0293	0.155
FAM84A	4.72	C6orf71	0.159
SLC39A8	4.689	GPLD1	0.164
LTB4R2	4.59	TRIB1	0.168
RP3-402G11.12	4.375	DKFZP586A0522	0.176
RPS19	4.354	MBNL2	0.177
PTAFR	4.323	SERPINA4	0.185
TCF15	4.236	FGD4	0.186
FTL	4.157	CYP3A5	0.19
LBP	4.083	CYP3A4	0.195
SAA3P	4.069	DHRS1	0.195
TGM2	4.062	C10orf65	0.202
BAIAP2L2	4.039	SLC25A15	0.203
SOX15	3.996	JUN	0.204
DLG4	3.995	RCL1	0.204
SLC35C1	3.919	ZGPAT	0.204
ELMOD2	3.911	DSIPI	0.207
DDX54	3.9	KLF6	0.209
AMBP	3.876	CYP26A1	0.21
SCUBE1	3.874	NDUFS1	0.213
SFXN4	3.865	AASS	0.218
TAOK2	3.782	CYP4A11	0.218
VWA1	3.779	TMEM16A	0.22
SRCRB4D	3.744	BAAT	0.221
ATF5	3.709	HGD	0.221
BBC3	3.678	PSMAL	0.232

Table 3a. Enriched GO Categories of Genes Up-Regulated in Donor Livers *versus* Controls

List Name	Description	Total Probes	Expected By Chance	Actual	Enrichment	P-Value
Regulation of JNK cascade	Any process that modulates the frequency, rate or extent of signal transduction mediated by the JNK cascade	37	1.06	8	7.51	0.00000859
DNA damage response, signal transduction by p53 class mediator	A cascade of processes induced by the cell cycle regulator phosphoprotein p53, or an equivalent protein, in response to the detection of DNA damage	44	1.27	8	6.32	0.00003298
DNA damage response, signal transduction resulting in induction of apoptosis	A cascade of processes initiated by the detection of DNA damage and resulting in the induction of apoptosis (programmed cell death)	48	1.38	7	5.07	0.00042682
Regulation of binding	Any process that modulates the frequency, rate or extent of binding, the selective interaction of a molecule with one or more specific sites on another molecule	59	1.70	7	4.12	0.00150276
Stress-activated protein kinase signaling pathway	A series of molecular signals in which a stress-activated protein kinase (SAPK) cascade relays one or more of the signals	136	3.91	15	3.83	0.00000995
JNK cascade	A cascade of protein kinase activities, culminating in the phosphorylation and activation of a member of the JUN kinase subfamily of stress-activated protein kinases	130	3.74	14	3.74	0.00002546
DNA damage response, signal transduction	A cascade of processes induced by the detection of DNA damage within a cell	115	3.31	12	3.63	0.00012753
Induction of apoptosis by intracellular signals	Any process induced by intracellular signals that directly activates any of the steps required for cell death by apoptosis	82	2.36	8	3.39	0.00252772

Table 3b. Enriched GO Categories of Genes Down-Regulated in Donor Livers *versus* Controls

List Name	Description	Total Probes	Expected by Chance	Actual	Enrichment	p-Value
Heterocycle metabolic process	The chemical reactions and pathways involving heterocyclic compounds, those with a cyclic molecular structure and at least two different atoms in the ring (or rings)	146	3.93	17	4.33	0.00000050
Monocarboxylic acid metabolic process	The chemical reactions and pathways involving monocarboxylic acids, any organic acid containing one carboxyl (COOH) group or anion (COO-)	396	10.65	41	3.85	0.00000000
Fatty acid metabolic process	The chemical reactions and pathways involving fatty acids, aliphatic monocarboxylic acids liberated from naturally occurring fats and oils by hydrolysis	283	7.61	29	3.81	0.00000000
Aromatic compound metabolic process	The chemical reactions and pathways involving aromatic compounds, any organic compound characterized by one or more planar rings, each of which contains conjugated double bonds and delocalized pi electrons	211	5.68	20	3.52	0.00000142
Sterol metabolic process	The chemical reactions and pathways involving sterols, steroids with one or more hydroxyl groups and a hydrocarbon side-chain in the molecule	147	3.95	13	3.29	0.00018779
Electron transport	The transport of electrons from an electron donor to an electron acceptor	599	16.11	48	2.98	0.00000000
Alcohol metabolic process	The chemical reactions and pathways involving alcohols, any of a class of alkyl compounds containing a hydroxyl group	541	14.55	39	2.68	0.00000005
Glucose metabolic process	The chemical reactions and pathways involving glucose, the aldohexose gluco-hexose, D-glucose is dextrorotatory and is sometimes known as dextrose	195	5.25	14	2.67	0.00090344

regulated genes in GO biological process categories, showing that apoptosis and stress activated protein kinase activities are the most affected pathways by up-regulated genes (Table **3a**), whereas down-regulated genes are involved in metabolic pathways and electron transport (Table **3b**). At least 30 mitochondrial enzymes, involved in oxidative chain, are from 2 to 5 folds down-regulated, in deceased DL, if compared to CL (Table **4**). Comparison between TL and CL samples demonstrated that 855 genes were dysregulated in TL versus CL. Condition tree from hierarchical clustering of these genes (Fig. **1**) clearly shows that about 1/3 of these genes were already dysregulated in DL if compared to controls: 182 genes, (mainly involved in oxidoreductase, electron transport and metabolic activity), were already down-regulated in DL group versus CL group and 135 genes, (involved in inflammatory pathways and cell adhesion), were already up-regulated in DL group versus CL group.

In conclusion, the present study demonstrated that: 1) 900 genes were dysregulated in DL if compared to controls; 2) 855 genes were dysregulated in TL if compared to CL. At least 400 of these genes were already dysregulated in DL.

A comparison of expression data between biopsies of living donors and livers from sudden death individuals is shown in the supplemental section (Supplementary file **2**).

Briefly, even though 217 genes are differentially expressed with fold change >1.75 and p<0.01 between livers

Table 4. Oxidative Phosphorilation Genes Down-Regulated in Donor Livers *versus* Controls

Gene Name	Gene Bank	Description	Fold Change DL *vs*. CL
AASS	AK023446	aminoadipate-semialdehyde synthase	0.218
ACOX1	BF435852	acyl-Coenzyme A oxidase 1, palmitoyl	0.296
ACOX3	BF055171	acyl-Coenzyme A oxidase 3, pristanoyl	0.534
ALDH5A1	NM_001080	aldehyde dehydrogenase 5 family, member A1	0.336
CAT	AW015521	catalase	0.266
CHDH	AA609488	choline dehydrogenase	0.493
COX15	AF026850	COX15 homolog, cytochrome c oxidase assembly protein (yeast)	0.358
COX7A2L	NM_004718	cytochrome c oxidase subunit VIIa polypeptide 2 like	0.547
CYP26A1	NM_000783	cytochrome P450, family 26, subfamily A, polypeptide 1	0.21
CYP2B7	M29873	cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene	0.349
CYP3A4	AV650252	cytochrome P450, family 3, subfamily A, polypeptide 4	0.195
CYP3A43	NM_022820	cytochrome P450, family 3, subfamily A, polypeptide 43	0.305
CYP3A5	AW964006	cytochrome P450, family 3, subfamily A, polypeptide 5	0.19
CYP3A7	AF315325	cytochrome P450, family 3, subfamily A, polypeptide 7	0.297
CYP4A11	BC041158	cytochrome P450, family 4, subfamily A, polypeptide 11	0.218
DAO	NM_001917	D-amino-acid oxidase	0.499
FLJ22378	NM_025078	hypothetical protein FLJ22378	0.524
HAO2	NM_016527	hydroxyacid oxidase 2 (long chain)	0.322
IBRDC2	AI953847	IBR domain containing 2	0.502
IVD	NM_002225	isovaleryl Coenzyme A dehydrogenase	0.488
KMO	NM_003679	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	0.291
NDUFA2	BC003674	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa	0.514
NDUFB7	NM_004146	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	0.376
NDUFB8	AA723057	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	0.455
NDUFV1	AF092131	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	0.504
NISCH	NM_007184	nischarin	0.549
PAOX	AI743990	polyamine oxidase (exo-N4-amino)	0.523
PIPOX	AF136970	pipecolic acid oxidase	0.437
UQCRB	BC005230	ubiquinol-cytochrome c reductase binding protein	0.437
ZDHHC4	NM_018106	zinc finger, DHHC domain containing 4	0.556

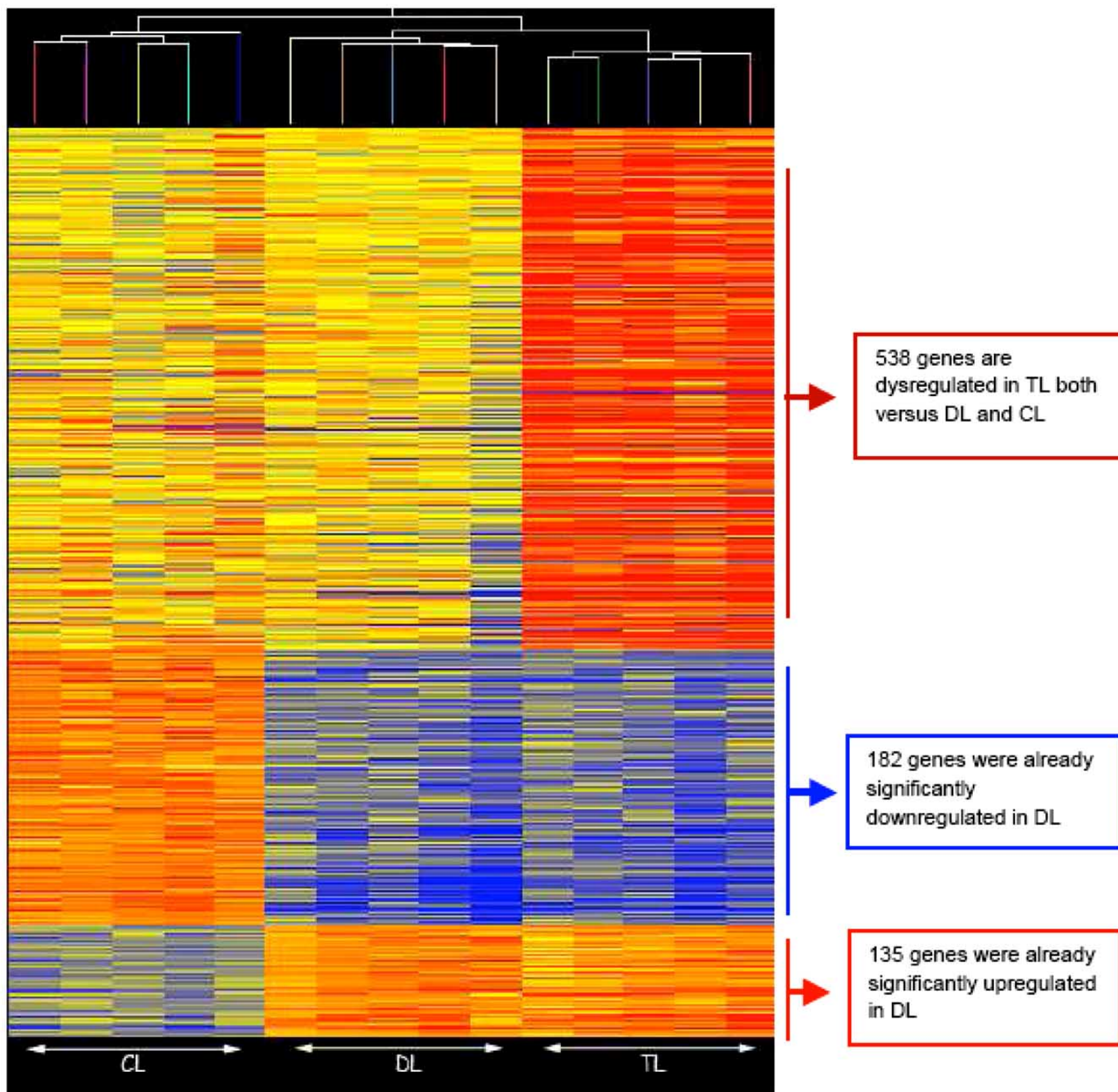

Fig. (1). Condition tree from hierarchical clustering of genes differentially expressed between TL and CL groups.

Samples from each group are well clustered together according to the expression level of these genes. The figure shows that many genes (in the lower part of the graphic), differentially expressed between TL and CL, were already dysregulated in the DL. Therefore their dysregulation might be due to brain death more than to ischemia/reperfusion injury. The genes in the upper part of the tree are dysregulated only in TL group both versus CL and DL samples.

Genes were considered differentially expressed with $p < 0.01$ using ANOVA test with Benjamini and Hochberg false discovery rate correction.

CL=Control livers, DL=Donor livers, TL=Transplanted livers.

from living donors and livers from deceased subjects (CL) only 54 (63 probe sets) out of these genes are included in the set of 900 genes we found differentially expressed in DL *vs*. CL.

Quantitative RT-PCR analysis of 26 genes, chosen among the most dysregulated genes, was carried out in order to validate the results of microarray analysis. The RT-PCR experiments confirm the microarray data for these genes. Correlation between quantitative RT-PCR and microarray data was satisfactory for all the tested genes ($r > 0.85$) (Table **5**).

DISCUSSION

Clinical studies on humans have shown that allograft from unrelated living donors have better graft function and survival than allograft from deceased donors [19-21]. This difference could be attributed to the pathophysiological changes derived from brain death and donor condition more than to the influence of cold ischemia times [22-24].

In this study we have demonstrated that brain death, together with other factors related to donor condition (shock, intensive care treatment, parenteral nutrition, etc.) causes the dysregulation of at least 900 genes in human liver tissue. The

Table 5. Comparison between Quantitative Real Time PCR and Microarray Data

Probe Set	Gene Name	Microarray Fold Change DL/CL	RT Fold Change DL/CL
205364_at	ACOX2 acyl-Coenzyme A oxidase 2	0.27	0.27
231587_at	APOC3 apolipoprotein C-III	9.51	14.25
209186_at	ATP2A2 ATPase, Ca++ transporting	5.48	1.90
228876_at	BAIAP2L2 BAI1-associated protein 2-like 2	4.04	3.50
212952_at	CALR calreticulin	9.41	21.75
204093_at	CCNH cyclin H	1.06	0.90
220046_s_at	CCNL1 cyclin L1	0.47	0.30
228766_at	CD36 CD36 molecule	0.13	0.25
213279_at	DHRS1 dehydrogenase/reductase	0.20	0.28
1555612_s_at	G6PC glucose-6-phosphatase	0.13	0.08
210328_at	GNMT glycine N-methyltransferase	0.12	0.30
215554_at	GPLD1 glycerol-3-phosphate dehydrogenase 1	0.16	0.28
241945_at	HECTD1 HECT domain-containing 1	0.15	0.34
201466_s_at	JUN jun oncogene	0.20	0.18
205222_at	LBP lipopolysaccharide binding protein	4.08	6.55
203675_at	NUCB2 nucleobindin 2	3.95	3.90
206278_at	PTAFR platelet-activating factor receptor	4.32	7.80
210479_s_at	RORA RAR-related orphan receptor A	0.14	0.33
222226_at	SAA3P serum amyloid A3 pseudogene	4.07	6.05
213874_at	SERPINA4 serpin peptidase inhibitor	0.19	0.20
222705_s_at	SLC25A15 solute carrier family 25	0.20	0.65
215223_s_at	SOD2 superoxide dismutase 2	2.04	2.40
217040_x_at	SOX15SRY (sex determining region Y)-box 15	4.00	5.50
207306_at	TCF15 transcription factor 15 (basic helix-loop-helix)	4.24	7.45
201042_at	TGM2 transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	4.06	4.65
239818_x_at	TRIB1 Tribless homolog 1 (Drosophila m.)	0.17	0.10

validation study (Supplementary file 2) demonstrates that 54 of them (6/%) might be affected by the choice of deceased tissues, as they are already dysregulated between CL and living donor tissues. Subtraction of these genes from the set of 900 genes does not affect at all the following considerations about injuries occurring in donor livers. Up-regulated genes are mainly involved in immune response, cytoskeletal remodelling, inflammation, apoptosis and cell adhesion. Down-regulated genes are mostly involved in mitochondrial activities and metabolism, being members of metabolic pathways of aminoacids, such as Gly, Ser and Cys, fatty acids (*HNF4alpha*) and vitamins. Metabolism might be also affected by starvation and stress, due to a long stay in intensive care unit. Molecular and cellular alterations triggered by brain death itself may significantly alter both early and long-term results of transplantation if compared to organs harvested from living donors [25].

Apoptosis induction and increased expression of apoptosis related proteins were observed in hepatocytes from brain dead animals [26]. A recent study on molecular changes induced in the heart by brain death [27], evaluates the variation in the expression levels of 5 genes involved in apoptotic processes: *BAX*, *BCL2*, *CASP3*, Cytochrome C (*CYCS*) and *FAS* and one gene induced by hypoxia (*HIF1A*) concluding that brain death mainly induces the expression of 3 out of these genes: *BAX*, *FAS* and *CASP3* involved in apoptosis activation. *HIF1A* is not significantly induced, excluding hypoxic damages. Our results show that *BAX* and *FAS* are induced in liver samples from deceased donors. Moreover in our experiments the anti-apoptotic *BCL2* gene is induced much more than in the heart as like as *HIF1A*, indicating that hypoxic injuries are already present in DL.

Our study unraveled that in deceased DL at least 30 mitochondrial enzymes, involved in oxidative chain, are from 2 to 5 fold down-regulated if compared to CL (Table **4**). It is known that during ischemia oxidative phosphorylation and ATP level decrease producing ischemic damage. The

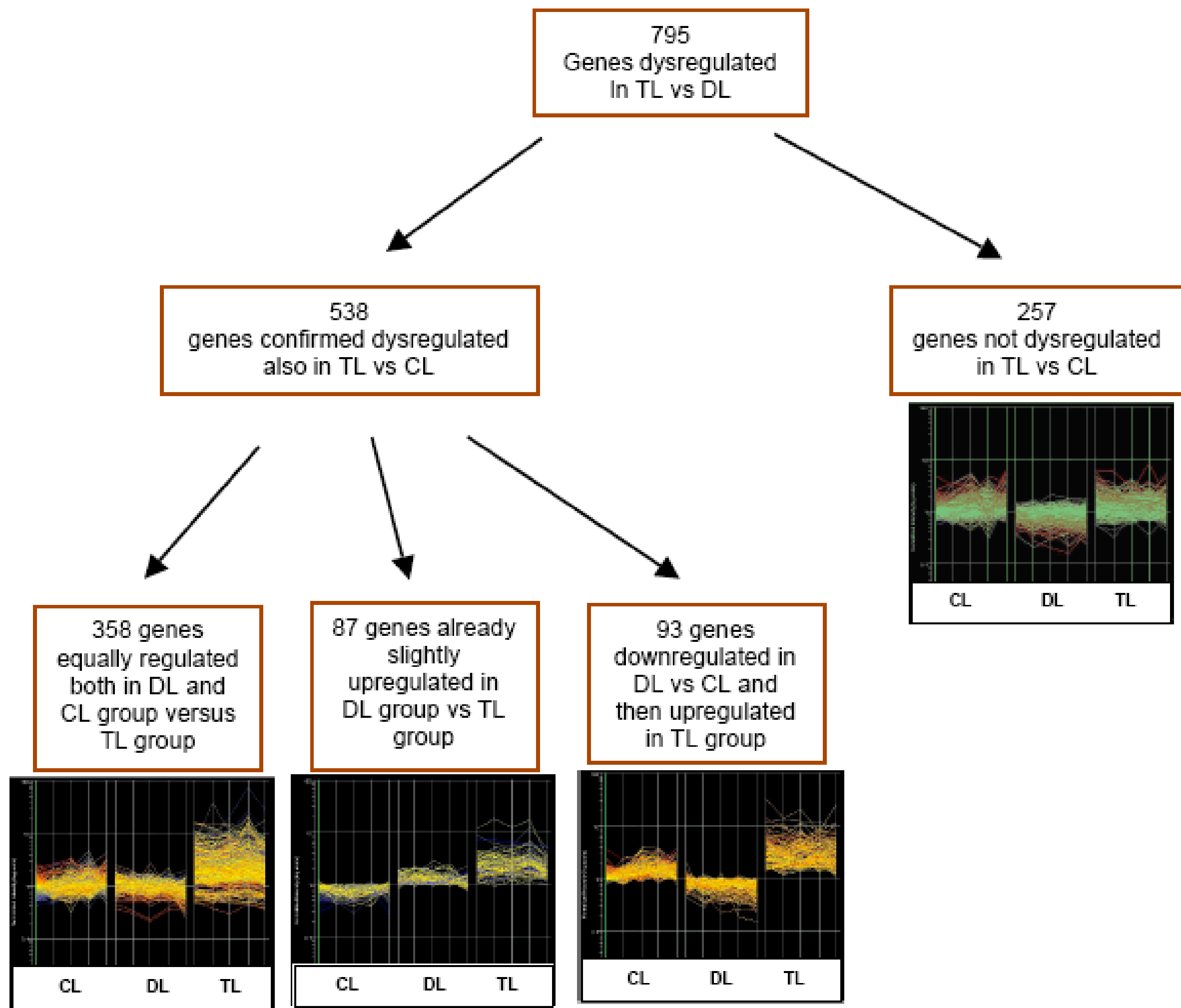

Fig. (2). Expression profiles of 795 genes previously found dysregulated in livers after transplant.

The figure shows on the left the expression profiles of 538 genes found dysregulated in a previous study, in TL *versus* DL, and now confirmed as dysregulated in TL if compared to CL. Detailed analysis of these genes, dysregulated both *versus* DL and CL, shows three different behaviors:

- 358 genes were normoregulated between DL and CL groups;

- 87 genes were already slightly up-regulated in DL group.

- 93 genes were down-regulated in DL group versus CL group and highly up-regulated after transplantation in TL group.

On the right side of the figure are shown expression profiles of 257 genes dysregulated in TL *versus* DL, but not confirmed as dysregulated in TL when compared to CL.

Genes were considered differentially expressed with $p < 0.01$ using ANOVA test with Benjamini and Hochberg false discovery rate correction.

CL=Control livers, DL=Donor livers, TL=Transplanted livers.

situation might be deeply compromised if the expression of oxidative enzymes is down-regulated.

A detailed analysis of 795 genes previously found dysregulated in TL when compared to DL [3], in the new perspective of the comparison between transplanted and control liver tissues, shows that about 250 genes, mainly involved in angiogenesis, lipid metabolism, growth, cell cycle, were not confirmed as dysregulated in our study when compared to CL (Fig. **2**). For these genes we hypothesize that their dysregulation is due to events related to brain death instead of IRI. However many genes reported as dysregulated in the previous study are dysregulated even when TL are compared to CL: 87 of them were already slightly up-regulated in DL group. These genes are mainly apoptotic regulators, proteolytic enzymes, chemokines, cytokines and stress responsive genes. Ninetythree more genes, down-regulated in DL, are essentially involved in growth, angiogenesis, mitosis initiation, cell cycle regulation and metabolism. We demonstrate that c-*FOS* and *c-JUN* are down-regulated by brain death causing inhibition of cell proliferation and *VEGF* signaling (angiogenesis). This might explain the better graft function and survival of allograft from living donors if compared to

Table 6. 15 Out of 78 Genes Predictive for IPGF, Already Dysregulated in Donor Livers *versus* Controls

Gene Name	Gene Bank	Description	Fold Change DL *vs*. CL
SOD2	R34841	Superoxide dismutase 2, mitochondrial	7.177
FCAMR	AW028140	FKSG87 protein	2.332
STCH	NM_006948	Stress 70 protein chaperone, microsome-associated, 60kDa	2.161
SOD2	BF575213	Superoxide dismutase 2, mitochondrial	1.898
ADAMTS1	AF060152	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	1.892
VNN2	NM_004665	Vanin 2	1.874
OSGIN2	BC031054	Chromosome 8 open reading frame 1	1.807
RAB31	BF510937	Homo sapiens transcribed sequences	1.802
IL-7	NM_000880	Interleukin 7	1.708
FLJ22684	NM_025048	Hypothetical protein FLJ22684	1.635
IFI16	AF208043	Interferon, gamma-inducible protein 16	1.62
NCOA7	AL035689	Nuclear receptor coactivator 7	1.564
LCP1	AW205969	Homo sapiens transcribed sequences	1.54
MAP4K4	NM_017792	Hypothetical protein FLJ20373	0.528
BAAT	NM_001701	Bile acid Coenzyme A: amino acid N-acyltransferase	0.221

deceased donors. All these genes are very highly up-regulated after transplantation and reperfusion.

In addition to the genes already found dysregulated in the previous study and confirmed in this study, we found 317 additional genes differentially expressed between TL and CL (Fig. **1**); these genes were not detected in our previous study [3] because they were already dysregulated in DL, therefore no differences between DL and TL group were detectable. In detail, 135 genes were equally up-regulated in DL and TL group versus controls. They are mainly anti-apoptotic genes, *NFKB* subunits, *BCL2*, *BCLXL*, cell adhesion molecules. 182 genes were equally down-regulated in DL and TL group versus controls. Most of them are involved in fatty acid, glucose and aminoacid metabolism and electron transport activities. Thus, inflammatory processes and oxidative phosphorylation activities are impaired in the liver since the pre-explant period, and continue to be impaired after transplant and reperfusion.

Functional analysis of the remaining genes dysregulated in TL group versus CL group completely confirms the results of our previous study [3]. Many authors agree with the idea that understanding molecular bases of graft failure is crucial to identify therapeutic targets able to improve transplant performance [28]. In this respect, a recent study identifies 78 classifier genes whose dysregulation after liver transplantation is able to predict initial poor graft function (IPGF) [29]. Our study demonstrated that 15 of these genes resulted already dysregulated in DL (Table **6**); molecular pathways involved in IPGF might be compromised before the organ is explanted and preventing these mechanisms in the donors possibly results in better graft function.

Recently De Jonge *et al.* [4] have been carried out in human OLT a study which analyzes the differential gene expression between donor baseline biopsies and post-reperfusion biopsies in two groups of liver transplantations: from living donors and from deceased donors. The authors find a large number of genes differentially expressed in both graft types following reperfusion when compared to the pre biopsies, more in the living transplant than in the deceased one. Among the group of genes differentially expressed in post-reperfusion biopsies of both groups they find a significant up-regulation of genes involved in inflammatory and immune processes, both in deceased and in living donor grafts.

CONCLUSIONS

Brain death and intensive care injuries induce stress in DL, affecting liver gene transcriptional profile both in donors and in recipients, and many genes dysregulated in TL *versus* CL are already dysregulated in DL before transplantation. On these bases we hypothesize that the dysregulation of these genes, mainly involved in inflammatory pathways, cell adhesion and electron transport, might affect graft function and organ survival in OLT. The insight of these mechanisms is crucial for the identification of therapeutic targets, aiming to improve OLT performances.

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ABBREVIATIONS

BD = Brain death

CL = Control Livers

DL = Donor livers

EDR = Expected Discovery Rate

GO = Gene Ontology

IRI = Ischemia Reperfusion Injury

OLT = Orthotopic Liver Transplantation

RMA = Robust Multiarray Analysis

TL = Transplanted Livers

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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