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PhD THESIS

- ABSTRACT -

LASER MICRODISSECTION AND GENE EXPRESSION PROFILING OF HEPATOCELLULAR CARCINOMA

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CONTENTS

ABBREVIATIONS

A	Adenine
AFP	Alpha fetoprotein
ASO	Assay Specific Oligo
C	Cytosine
CCd	Cholangio-cellular differentiation
cDNA	Complementary DNA
DASL	cDNA-mediated Annealing, Selection, Extension, and Ligation
Diff score	Differential score
DNA	Deoxyribonucleic Acid
DPV	Detection p-value
DSO	Downstream-Specific Oligo
FF	Fresh/frozen
FFPE	Formalin fixed, paraffin- embedded
FGF	Fibroblast growth factor
G	Guanine
GO	Gene ontology
GTP	Guanosine triphosphate
H&E	Hematoxylin and eosin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
ICS	Illumina iScan ™ Software
IGF	Insulin-like growth factor
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
NASH	Non-alcoholic steatoheptitis
OLT	Orthotopic liver transplantation
OPN	Osteopontin
PCR	Polymerase chain reaction
PEN	Polyethylene naphthalate
qPCR	Quantitative PCR
RIN	RNA integrity number
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription-polymerase chain reaction
TACE	Transarterial chemoembolization
TGF-β	Transforming growth factor-beta
U	Uracil
USO	Upstream-Specific Oligo
VEGF	Vascular endothelial growth factor
WG-	Whole genome

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer in men and the seventh in women and is the third most common cause of death from cancer worldwide [http://globocan.iarc.fr]. The overall incidence of HCC remains high in developing countries and is rising in many of the industrialized countries [Shariff et al 2009], making it a field of interest for researchers everywhere.

The only curative treatment for HCC is liver transplantation [El-Serag and Mason, 1999; Llovet et al 2003; Geschwind et al 2003], but shortage of donor organs may result in an increased time that patients must remain on the waiting list [Stippel et al 2003]. Thus, interventional radiological therapy, especially transarterial embolisation (TACE) is rising particular interest and importance as it is ''bridging the time to transplant'' [Graziadei et al 2003; Yao et al 2005; Porrett et al 2006; Lencioni et al 2005; Decaens et al 2005]. Due to conflicting results of TACE there is still an ongoing discussion concerning its efficiency [Porrett et al 2006; Pompili et al 2005; Ravaioli et al 2004; Sotiropoulos et al 2005; Veltri et al 1998; Majno et al 1997]. It was also suggested that in some patients TACE could induce a more aggressive form of HCC, characterised by a biliary phenotype [Lee et al 2006; Zen et al 2011].

Also, the pathogenesis of HCC is still not well understood. For identifying molecular and genomic mechanisms involved in liver tumorigenesis, microarray technology has been extensively applied, several gene expression profile studies being performed in the last 10 years [Maass et al 2010]. One of the downsides of the technology available until recently was that it was limited to RNA extracted from fresh/frozen (FF) tissue or cell cultures. Recent advances have made it possible to obtain good quality RNA from formalin-fixed paraffin embedded (FFPE) tissue, allowing access to a virtually limitless archival resource to be available for retrospective and long term prospective clinico-pathological studies.

The Illumina Inc. specially designed gene expression profiling method DASL (cDNA-mediated Annealing, Selection, Extension and Ligation), has been developed for the analysis of fragmented RNA samples [April et al 2009; Bibikova et al 2004a; Bibikova et al 2004b], that are usually a consequence of formalin fixation.

Laser-capture microdissection is a technique that allows the isolation of specific cell populations [Blatt and Srinivasan 2008] or of specific microscopic areas of interest from tissue samples. This allows the selective evaluation of gene expression of targeted cell clusters [Tachikawa and Irié et al 2004], especially in a very heterogeneous environment as the malignant tissue.

I have chosen this particular subject because it is part of the current fields of interest in research. Furthermore, there is no publication in the up to date literature that mentions the use of FFPE liver samples, in combination with laser microdissection and whole genome DASL assay, conferring an element of originality to my study. Unrevealing the genomic signatures of HCC can be used to help characterize the molecular changes responsible for its development and it could be extremely valuable in the development of new tumour markers novel therapeutic targets.

I want to mention that this work is the result of a long lasting care from the people who guided me and tought me everything I know and next to whom I worked and learned to love the great field of Pathology. I will always keep them in my heart and I want to deeply thank Professor Emil Pleşea, Doctor Alberto Quaglia and Professor Cristiana Simionescu and to the rest of the people that helped me along the way.

Last, but not least, I want to send all my love to my parents, because without their help and support I would not be anything of what I am today...

BACKGROUND

Chapter I – "Hepatocellular carcinoma" describes in detail the latest data regarding the epidemiology of hepaatocellular carcinoma, its aetiology, clinical features, and aspects of imaging and histopathology.

Chapter II – New approaches on hepatocarcinogenesis" presents a novel approach to the understanding of hepatocarcinogenesis with particular reference to the signalling pathways recently shown to be involved in the pathogenesis of this disease.

Chapter III – "Treatment and pre-transplant ablative therapies of hepatocellular carcinoma" deals with the currative, ablative and molecular therapies of HCC, with details on advantages and disadvantages of their use.

PERSONAL CONTRIBUTION

AIM OF STUDY

The study was designed for answering the following question:

- Is there any difference in the gene expression profile of hepatocellular carcinoma when compared to the background liver?
- Is there any difference in the gene expression profile of the classical type of HCC when compared to the cholangiocellular differentiation areas, after TACE?

MATERIALS

Twenty-nine cases of HCC which underwent liver transplantation or resection preceded by TACE (Doxorubicin 40mg/m2), between 2008 and 2010, were retrieved from the archive of the Histopathology Laboratory, Institute of Liver Studies, King's College Hospital, London. Before the TACE treatment, all nodules were radiologically diagnosed to be HCC according to the European Association for the Study of the Liver criteria [Bruix et al 2000] for concordant imaging of nodular arterialized lesions with portal venous washout.

METHODS

GROSS EXAMINATION

Livers removed during surgery were received fresh, and they were sliced into parallel sections at approximately 1-cm intervals. Twenty-eight of the cases underwent liver transplantation and only one was treated with right hepatectomy.

Standard examination procedure included macroscopic evaluation, assessing the number, size, and location of the different tumour nodules. They were measured in 3 dimensions, the maximum diameters being recorded. The livers were re-examined after formalin fixation, and the tumours were sampled more extensively. Formalin-fixed tissue was embedded in paraffin, and the sections were stained with hematoxylin and eosin (H&E) as part of the routine histological assessment.

MICROSCOPIC EXAMINATION

Histology sections with samples of each tumour were reviewed using an Olympus microscope. Liver parenchyma in which hepatocytes maintained a liver cell plate structure with staining nuclei, were considered viable. Areas of non-viable tissue were identified as areas of confluent coagulative necrosis of liver parenchyma, fibrous scarring and/or granulation tissue.

Most of the post-TACE HCCs examined in this study had microscopic foci of viable hepatocellular carcinomas, as well as non-viable lesional tissue with features of coagulative necrosis, fibrous scarring, inflammation and granulation tissue consistent with the effects of therapy. The cases without viable tumours were not included in further molecular studies.

The viable component was assessed in terms of tumour differentiation and presence of features suggesting combined hepatocholangiocellular differentiation, such as the formation of ductules, glandular structures or other combined growth patterns [Zen et al 2011]. The background liver, in sections away from the tumours was also examined.

LASER MICRODISSECTION

Formalin-fixed and paraffin embedded (FFPE) samples were also cut at 5µm thickness using a Leica microtome. The sections were placed onto RNaze free polyethylene naphthalate (PEN) membrane coated slides and laser microdissected using the Leica LMD 6000 system.

The Leica LMD 6000 system runs a morphometry software which allowed the instantaneous calculation in µm2 of the selected areas for microdissection. An area of around 10.000.000 µm2 was microdissected in multiple cuts under low magnification. Microdissected tissue was collected in 1.5 ml microfuge caps.

PURIFICATION OF TOTAL RNA FROM MICRODISSECTED FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE SECTIONS

The next step was to purify total RNA from the microdissected formalin fixed, paraffin-embedded tissue (FFPE) sections using the QIAGEN RNeasy® FFPE Kit for purification of total RNA, following the protocol set by the manufacturer.

Overview (adapted from QIAGEN RNeasy® FFPE Kit handbook)

The RNeasy FFPE procedure uses specially optimized lysis conditions that allow total RNA to be purified from FFPE tissue sections. DNA contamination is removed during the DNase digestion step, together with any highly fragmented molecules that might exist in the samples.

In the beginning of the process all paraffin was removed from the FFPE tissue sections by treatment with xylene. One hundred percent ethanol was then added to extract any residual xylene from the sample. Next, samples were incubated in an optimized lysis buffer, which contained proteinase K, to release RNA from the sections. A short incubation at a higher temperature partially reversed formalin crosslinking of the released nucleic acids, improving RNA yield and quality. The DNase treatment that followed was designed to eliminate all genomic DNA, including very small fragments that could have been present in FFPE samples. Next, the lysate was mixed with Buffer RBC. Appropriate binding conditions for RNA were created by adding ethanol. The samples were then transferred to an RNeasy MinElute spin

column, which contained a membrane for binding total RNA, contaminants being efficiently washed away. RNA was then eluted in 14 µl of RNase-free water.

YIELD AND QUALITY EVALUATION OF TOTAL RNA

The concentration of the purified RNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies; Wilmington, DE) by measuring the absorbance at 260 nm (A260) and 280 nm (A280).

An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 =1= 40 µg/ml). The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. Pure RNA has an A260/A280 ratio of 1.8–2.0.

Purified total RNA samples were stored frozen at -80°C until needed for quality control (QC) analysis and subsequent gene expression profiling.

QUALITY CONTROL ANALYSIS

The quality control (QC) was done with the help of Qubit® Quantitation Platform and Agilent 2100 Bioanalyzer, representing fluorescence-based and an electrophoretic assay respectively.

Fluorescence-based quantitate assay - Overview (adapted afrer Qubit® fluorometer instruction manual)

This process uses an RNA quantitation kit to quantitate RNA samples for the Whole-Genome DASL Assay.

Because the Quant-iT™ assay kit used dyes that are selective for RNA, contaminants in the sample should not affect the quantitation. Illumina recommends using a fluorometer rather than a spectrophotometer, because fluorometry provides RNA-specific quantification, while spectrophotometry might also measure DNA, the resulting yield values being not as accurate. Qubit® Quantitation Platform was used based on its highly sensitive fluorescence-based assays.

Electrophoretic assay - Overview (addapted from agilent 2100 bioanalyzer, 2100 expert user's guide)

The electrophoretic assays was run using the Agilent 2100 Bioanalyzer and the data were interpreted with the help of 2100 expert software.

The electrophoretic assays are based on the principles of traditional gel electrophoresis, but a chip format is being used. Every chip is composed from several wells for the samples and the gel and one well for an external standard (ladder). The ladder contains components of known sizes, thus a standard curve of migration time versus fragments size can be plotted.

The size is calculated using the migration times measured for each fragment in the sample. The ladder area is used for quantitation, meaning that the area under the ladder is compared with the sum of the sample peak areas, not taking into consideration area under the "lower" marker. The marker solution contains a 50 bp DNA fragment and it is displayed as the first peak in the electropherogram.

When the wells and channels are filled, the electrodes are connected to a power supply and the chip acts as an integrated electrical circuit. The voltage gradient electrophoretically

drives the charged RNA biomolecules, smaller fragments migrating faster than larger ones. In this way, the molecules are separated by size.

As dye molecules intercalate into the RNA strands, these complexes can be detected by laser-induced fluorescence. In this way, data is translated into band images and electropherograms. The integrity of the total RNA sample is determined with the help of the ribosomal ratio and the RNA integrity number (RIN). Numbers from ’1’ to ’10’ are used to label the samples, ’10’ meaning no degradation products and ’1’ being assigned for a completely degraded sample.

WHOLE GENOME DASL ASSAY

The integrity of nucleic acids is very important in microarray analysis. FFPE tissue RNA is often degraded and chemically modified [Masuda et al 1999; Srinivasan et al 2002]. The Illumina Inc. gene-expression profiling method DASL (cDNA-mediated Annealing, Selection, extension and Ligation), is specially designed for analysis of fragmented RNA samples [April et al 2009; Bibikova et al 2004a; Bibikova et al 2004b].

Overview (whole-genome gene expression DASL® assay guide)

The WG-DASL assay started by converting through reverse transcription reaction the total RNA into cDNA. This reaction used biotinylated oligo-dT18 and random primers. The biotinylated cDNA was annealed with assay-specific oligonucleotides (ASO) specially designed for a single contiguous 50 nucleotide sequence on each cDNA. These oligonucleotides are composed of two parts: an upstream-specific oligonucleotide (USO) containing a 3’ gene-specific sequence and a 5’ universal PCR primer, and a downstream-specific oligonucleotide (DSO) containing a 5’ gene-specific sequence and a 3’ universal PCR primer [Fan et al 2004]. The gene-specific sequence corresponds to a capture sequence on the BeadChip. A number of 47.000 oligonucleotide pairs (probes) were used, derived from the National Center for Biotechnology Information Reference Sequence Database (Build 36.2, Release 38). The ASOs were then annealed to the biotinylated cDNAs and the mixture was bound to streptavidin-conjugated paramagnetic particles for selection of the cDNA/oligo complexes. Polymerase extension of the USO and ligation to the corresponding DSO followed. The resulting products were PCR-amplified and labelled with a universal fluorescently labelled primer. The single-stranded labelled products were then hybridized on the complementary gene-specific sequence bead to Illumina Whole-Genome Gene Expression Human HT-12 v4 BeadChips and scanned with the iScan™ Reader.

Gene expression microarray data quality control

Quality control (QC) of data is an important step when performing any microarray gene expression study. Illumina Gene Expression BeadChips have internal control features to monitor data quality.

BeadStudio control features are either sample-independent or sample-dependent. The sample-independent metrics make use of oligonucleotides spiked into the hybridization solution. Poor performance measured by these controls could indicate a general problem with the hybridization, washing, or staining. The sample-dependent metrics are based on measurements from the actual sample of interest. Poor performance of these controls may indicate a problem related to the sample or labelling.

Normal variations in control plot values can arise due to incidental factors such as system setup, sample origin, and BeadChip type. These factors make it difficult to determine

data quality by comparison to a specified expected value for each QC metric. To minimize the influence of these factors, relative control values were used as QC criteria.

Relative comparisons of control values could be made by identification of outliers by comparison to current and historical data. Outlying samples for any given control metric can be quickly identified in BeadStudio by using the control summary plot and expanding the plots to view QC values for individual samples.

For enabling the sample outlier detection, the Illumina metric "Genes Detected" has been used.

BeadStudio calculates and reports a detection p-value (Genes Detected), which represents the confidence that a given transcript is expressed above the background defined by negative control probes.

This detection score determines whether a transcript on the array is called detected. A value below the user-defined p-value threshold of either 0.01 or 0.05 indicates a gene is detected.

All samples on a given BeadChip, prepared from the same sample source, should have a similar number of detected transcripts. Large extreme spikes/troughs in the plot would indicate samples that are outliers and may need to be repeated or excluded from further analysis.

STATISTICAL ANALYSIS

Data extraction and normalization

The illumina iScan™ software (ICS version 3.2) was used to extract and normalise the expression data (fluorescence intensities) for the mean intensity of all arrays. Normalization transforms the range of intensity values for a bead chip stripe to match a target range based upon a mapping file associated with each BeadChip type manifest and cluster files. ICS normalises the data in the *.idat file for a BeadChip section and generates a genotype call. The normalised data and genotype calls were saved in *.gtc file.

Gene array data analysis

The GenomeStudio™ Gene Expression Module v1.0 was used to analyse gene expression data using the intensity file from the scanned microarray images generated by the iScan™ System. This software can be used for gene analysis, for quantifying gene expression or for differential gene expression analysis that can determine the probability that gene expression levels have changed between two groups or samples.

This software averages values for each gene across samples, and algorithms automatically use replicates to provide estimates of relative mRNA abundance for detecting differential gene expression.

In brief, to identify differentially expressed genes, the following were applied: a detection p value < 0.01 and a differential score >13 (corresponding to a p – value < 0.05) under the Benjamini and Hochberg False Discovery Rate correction for multiple tests. In this research, where appropriate, groups of samples were used as biological replicates.

Normalization Methods & Algorithms

The Iillumina software package has normalization algorithms that adjust sample signals, to minimize the effects of non biological factors, such as differences in between arrays and chips. Firstly, a mathematically calculated "virtual" sample is created, that represents averaged probe intensities across all samples in the group. An average normalisation was used, where background was subtracted from sample intensities, before they were scaled, by a

factor equal to the ratio of the average intensity of the virtual sample, to the average intensity of the given sample. As a result, half of unexpressed targets would have negative signals after background subtraction.

Differential Expression Algorithms

To determine differences in gene expression between a group of samples (the condition group) and a reference group, the T-test was used. When the reference group or condition group contained at least 2 samples, the variance was estimated across replicate samples; otherwise the variance was calculated from the bead to bead variation. The t-test assumed equal variance.

A differential score (Diff Score) for each probe was calculated as follows:

$$DiffScore = (10sgn(I_{cond} - I_{ref})log_{10}(p)) \qquad I = intensity$$

The Diff Score is a transformation of the p-value that provides directionality to the p-value based on the differences between the average signal in the reference group and the comparison group.

- ***For a p-value of 0.05, Diff Score = ± 13***
- ***For a p-value of 0.01, Diff Score = ± 20***
- ***For a p-value of 0.001, Diff Score = ± 30***

For a given gene, Diff Scores of corresponding probes were averaged. To calculate a p value from the Diff Score, the following formula was used:

$$p = 1/(10[Diff\,Score/10(sgn(Icond - Iref)])$$

For multiple comparisons the Benjamini and Hochberg False Discovery Rate was applied to reduce chances of identifying genes as false positive or false negatives. The principle of this correction is that the p-value of each gene is ranked from the smallest to the largest. The largest p value is not modified. The second largest p-value is multiplied by the total number of genes (n) in the gene list, divided by its rank (n-1). If the result is less than 0.05, the gene is regarded as significant. The third largest p value is multiplied as before i.e corrected p-value = p-value (n/n-2). If the result is less than 0.05, it is regarded as significant. This process is repeated for all subsequent p-values.

Detection P-Value (DPV)

The detection p-value is the probability that the signal from a given probe is greater than the average signal from the negative controls. It is calculated with the equation:

$$DPV = 1 - R/N$$

R = rank of Z score of the analytical probes

N = number of negative controls

The Z score is calculated with the equation:

$Z_{ig} = I - \mu_i^{neg} / \sigma_i^{neg}$

μ_i^{neg} mean signal of negative controls on i^{th} sample and g^{th} gene

σ_i^{neg} standard deviation on i^{th} sample and g^{th} gene

When the samples were combined to form a group, the Z score was averaged. If the Z score for the probe intensity was smaller than the lowest negative control Z score, the p- value equalled 1. If the Z score of the probe intensity fell within the range of the Z score for the negative controls, the p-value was in the 0-1 interval. If the Z score of the probe intensity was greater than the largest negative control Z score, the p value equalled 0.

Questions to be answered

The statistical analysis of the Whole Genome DASL assay was specially designed for answering the following question:

- Is there any difference in the gene expression profile of hepatocellular carcinoma when compared to the background liver?
- Is there any difference in the gene expression profile of the classical type of HCC when compared to the cholangiocellular differentiation areas, after TACE?

The tumours belonging to one case were named HCC1, HCC2, ..., HCCx, where x was the maximum number of tumours microdissected for each case. A gene pool was created for the hepatocellular carcinoma, where one tumour for each case was included, since the difference in the number of samples for comparison was too large (34 HCCs vs. 19 background livers). The HCC gene expression profile was statistically analyzed in comparison to the gene pool created for the corresponding background livers (19 HCCs vs. 19 background livers). The average signal, detection p-value and differential score were calculated for each gene.

A different gene pool was created for the cholangiocellular differentiation, and this was compared to the classical type of HCC gene pool. The average signal, detection p-value and differential score were calculated for each gene.

RESULTS AND DISCUSSIONS

The results and discussions were divided into four sections, covering the clinico-pathological and bio-molecular aspects, including the RNA extraction and quality control assessment, and gene expression profiling data and their interpretation.

CLINICO-PATHOLOGICAL ASPECTS

Seven (24%) of the 29 patients included in the study were females and 22 (76%) males.

The age of the patients at the time of liver surgery ranged from 47 to 67 (mean = 58 y).

Regarding the aetiology of the tumours, 13 (45%) of them arose in the context of HCV infection, 5 (17%) were alcohol induced and 3 (10%) in HBV infection. We also found one HCC (3.6%) within the liver of a patient with Budd-Chiari Syndrome, 2 (7%) in the context of non-alcoholic steatoheptitis (NASH), 1 (3.5%) in the context of alpha-1 Antitrypsin deficiency and 2 (7%) following autoimmune conditions (Autoimmune Hepatitis and

Autoimmune Hepatitis/Primary Sclerosing Cholangitis Overlap Syndrome). Two cases (7%) had an unknown aetiology.

Eleven of the 29 patients included in the study had elevated pre-TACE alpha-fetoprotein serum levels (38%). They underwent between 1 and 4 cycles of TACE, with an average time between the treatment and the transplant procedure of 7.1 months (ranging from 1 to 86 months). At the present date, 27 (93%) of the patients are still alive, the overall mean survival being 35.7 months (range 19-77 months).

The majority of the livers (16) had the tumours in the right lobe (55.2%), 10 of them in both the right and left lobes (34.5%), and just 3 only in the left lobe (10.3%).

All the examined tumours were under 5 cm. Fifteen of the livers (52%) had a single tumour, 7 (24%) had 2 tumours, 2 (7%) had 3 tumours, and 5 (17%), 4 or more tumours.

From the total of 65 tumours examined, 51 (78.4%) had viable foci and 14 (21.6%) were wholly necrotic. Therefore, 7 (24%) of the 29 patients were excluded from further studies, due to the lack of viable tumour.

Extensive tumour necrosis was observed in 12 (41.4%) of the patients, the areas of non-viable tumour consisting of a combination of necrosis, fibrous scarring and granulation tissue.

Viable hepatocellular carcinoma showed a wide range of differentiation, from well to poorly differentiated. Twenty-four of the nodules (47%) identified were moderately differentiated hepatocellular carcinoma, 8 (15.5%) of them were well differentiated and 3 (5.9%) poorly differentiated. There were also tumours with biphasic differentiation, 13 (25.5%) of them showing moderately differentiated and one (1.96%) well differentiated HCC, with areas of poor differentiation, and also 2 (3.92%) moderately differentiated with small well differentiated areas. Macroscopic or microscopic vascular invasion was present in 13 of the livers examined (44.8%).

The phenotype of the tumours was mostly hepatocellular, but 7 (13.7%) of them showed a mixed phenotype, including glandular/pseudoglandular formation and cholangiocellular components. The aetiology of this mixed phenotype tumours was HCV infection in 4 of them, the others being 1 alcohol related, one Budd Chiari Syndrome and one NASH.

On the examination of the background liver, 26 (89.6%) were cirrhotic and only 3 (10.4%) cases had bridging fibrosis, short of cirrhotic transformation.

Assessment of tumour response after locoregional therapies is important in determining treatment success and in guiding future therapy [Vossen et al 2006].

The present study is in accordance with the up to date literature, 24.1% of the patients having no residual viable tumours at the histopathological examination of the liver explant, extensive tumour damage being achieved in 41.4% of our patients, and approximately 14% of them showing a mixed hepatocellular-cholangiocellular phenotype. The aetiology of this mixed phenotype tumours was in majority HCV infection, finding which requires further investigations for a possible correlation between the viral infection and the post-TACE cholangiocellular differentiation of HCC.

According to practice guidelines [Bruix and Sherman 2011] complete response to TACE is achieved in fewer than 2% of patients; the rate of objective response ranges from 16% to 60%; and extensive tumour necrosis occurs in more than 50% of patients [Bruix and Sherman 2011].

Yao et al presented a prospective study in 2005 that addressed the value of pretransplant locoregional treatment [Yao et al 2005]. Relevant down-staging was achieved in their cohort in 70% of the patients, and complete tumour necrosis was achieved in 44% of the patients, which is superior to the results published by Majno et al, Decaens et al and Zen et al [Decaens et al 2005; Majno et al 1997; Zen et al 2011]. Decaens et al evaluated two groups separately, the TACE group and the untreated control group, both consisting of 100 patients. They achieved a total or at least subtotal necrosis in 30% of the patients [Decaens et al 2005].

One of the first publications dealing with pretransplant TACE was published by Majno et al in 1997 [Majno et al 1997]. They started applying pretransplant TACE in the late 1980s as a substantial part of the pretransplantion treatment. In 54 patients who received TACE prior to OLT they found complete tumour necrosis in 27% and they achieved at least a relevant down-staging in half of their patients [Majno et al 1997]. In 2005 Sotiropoulos and colleagues reported similar results, with tumour regression in more than 50% of the patients [Sotiropoulos et al 2005].

In regard of tumour necrosis after TACE, Zen et al looked at 80 HCC nodules from 64 patients who underwent transplantation at the Institute of Liver Studies from 2004 to 2010. Their results were comparable to those reported over the last decade [Graziadei et al 2003; Porrett et al 2006; Decaens et al 2005; Ravaioli et al 2004; Majno et al 1997; Kim et al 2006; Wong et al 2004; Yao et al 2005; Sotiropoulos et al 2005; Bharat et al 2006; Cheng et al 2005].

Morisco et al [Morisco et al 2008] who studied the effect of different modalities of local ablation therapy on HCCs in 26 patients, found viable tumour in the majority of explanted livers, other studies reaching similar conclusions [Wong et al 2004; Marin et al 2009; Eguchi et al 2009]. In the series by Kim et al [Kim et al 2006] complete necrosis was noted in about a third of post-TACE HCC, with no significant difference in terms of number of tumours, tumour size, satellite nodules, capsular or vascular invasion, compared to non-treated tumours.

Histological assessment of tumour response has many limitations, even on specimens derived from surgical resections or transplantation. The tumour mass can be sectioned into thin slices, particularly after fixation and with use of metal bars to guide the scalpel or knife blade Even a thin, few mm thickness slice, is still of a much larger order of magnitude compared to the microscopic fields in which the relationships between residual tumour and the post-TACE changes are assessed.

Another limitation is to represent histologically the full tumour face. This can be achieved for tumours of round or oval shape in which the transverse diameter is up to about 20 mm. Multiple full face cross sections of the tumour could therefore be submitted individually in single conventional embedding cassettes which, despite the limitation related to the section thickness described above, would give a reasonable representation of the entire tumour surface. In such cases assuming the cut thickness is known and remains constant, and the histological representation of viable and non-viable tumour is maintained throughout the section thickness, it would be possible to calculate the tumour mass volume, and the proportional volumetry of viable and non-viable tumour, or even necrosis, fibrous scarring, granulation tissue and various areas of viable tumour differentiation, perhaps with the aid of 3D reconstruction software. A similar approach would be impractical for larger tumours, as whole tumour embedding would involve a large number of conventional tissue blocks, unless large “megablocks” are used [Cotoi et al 2012a].

Another aspect to take into account is the morphological definition of necrosis. Post-TACE HCC necrosis usually appears to the pathologist eyes as large area of tumour appearing as homogenous sheets of amorphous eosinophilic material, with cell ghosts in the background and often a retained reticulin and sinusoidal structure, which can be highlighted by a silver impregnation stain or immunohistochemistry for endothelial markers. The true state of morphologically “viable” tissue interspersed between areas of necrosis or in the vicinity however remains usually unknown. A previous study has show that peritumoural areas appearing as viable can be actually defunct when histochemical techniques are applied to test enzymatic function [Morimoto et al 2002].

The response of tumour to therapy could also consist of differentiation into a mature phenotype simulating the normal non-neoplastic counterpart. This has been observed in

hepatoblastoma [Saxena et al 1993], and in a mouse model of HCC regression and progression following c-mic activation/inactivation [Shachaf et al 2004]. In other words, tissue which appears non-neoplastic and or viable could be dead and/or neoplastic in various combinations.

Local ablation therapy is now being used for other sites besides liver, for primary or metastatic cancer, including lung and kidney. Most of the literature that I was able to access in relation to this topic is clinically orientated and does not give much information on the histological aspects of tissue response to treatment. No other histological changes of the tumours, apart from the ones associated with coagulative necrosis, have been reported on biopsy or resection specimens [Gebauer et al 2007; Margulis et al 2004; Steinke et al 2002].

RNA EXTRACTION AND QUALITY CONTROL ASSESSMENT

The microdissected tissue from the 22 cases with viable tumour underwent RNA purification from the formalin fixed, paraffin embedded tissue. The purified RNA was spectophotometrically analyzed. The average RNA concentration was 42.3 ng/μl (range: 5.4-178.1) with an average 260:280 ratio of 1.95 (range: 1.66-2.21).

Seven of the samples did not pass the quality control assessment and were not included in the WG-DASL assessment, thus the analysis being run on 20 of the initial 29 cases.

It is known that formaldehyde reacts with the nucleic acids in several ways. The formation of an N-methylol (N-CH2OH) followed by an electrophilic attack to form a methylene bridge between amino groups was speculated by Srinivasan et al [Srinivasan et al2002]. Masuda et al tried to prove this hypothesis using oligo RNA. We learn from their study that reactivity of the bases decreases in the following order: U<G<A/C, pointing out that the tertiary amino group is the first which is being targeted by formalin. [Masuda et al 1999] On this basis, McGhee and von Hippel concluded that the poly(A) tail of mRNA would be strongly modified by fixation. Thus, reverse transcription would not synthesize the best cDNA due to a non-proper annealing of the oligo (dT) to the poly(A) tail [McGhee et al 1977].

Another disadvantage for the cDNA synthesis is the degradation of RNA caused by formalin-fixation, meaning that the purified RNA from FFPE tissue might not contain both the poly(A) tail and the target area for PCR amplification [Masuda et al 1999]. This highly degraded RNA has proved not to be useful in the conventional microarray studies [Karsten et al 2002]. The Illumina WG-DASL assay kit uses random-priming in the cDNA synthesis step, especially for overcoming the downsides of formalin fixation, any unique regions of the gene being recognized by the probes, without limiting the targeting with optimal probes at the 3' end of the transcripts [Bibikova et al 2004b].

Several studies have been carried out in the past years using wide genome DASL assay on a number of different normal and pathological FFPE tissues, including breast, prostate, liver, colon and lung [Bibikova et al 2004a; Kibriya et al 2010; Bibikova et al 2004b; Hoshida et al 2008]. One of these studies conducted on samples from the colon found that sets of differentially expressed genes identified in FFPE samples resembled those identified from fresh-frozen samples, but with approximately 50% less genes detected in the assay using RNA purified from FFPE tissue [Bibikova et al 2004b]. Another study with highly reproducible intensity measurements, which demonstrated that gene expression profiling of RNAs from FFPE samples is possible, was run on prostate, colon, breast, and lung tissue. By using DASL assay and universal microarrays, despite the extensive degradation of the material, they demonstrated that DASL assay combines the advantages of array-based gene expression analysis with those of multiplexed qPCR [Bibikova et al 2004a]. In their data

interpretation, the importance of recognizing that the output of the DASL assay reflects the extended and ligated query oligonucleotide pool was highlighted. The measurement of gene expression is done indirectly and it depends on the "labelling competition" in the PCR amplification. Thus, changes in hybridization signal may not reflect changes of the number of transcripts in the most accurate way [Bibikova et al 2004a].

Hoshida at el used RNA extracted from macrodissected FFPE tissue samples of hepatocellular carcinoma and adjacent liver to run a wide genome DASL assay. They obtained high quality data from samples of 90% of their patients, including the ones that were in storage for more than 24 years [Hoshida et al 2008].

In 2010 Kibriya et al conducted a study on breast cancer tissue using WG-DASL assay, and compared the gene expression profile of FFPE and fresh frozen (FF) tissue. Similarities between FFPE and FF samples according to gene ontology classification suggested that FFPE can be successfully used for identifying groups of genes that may be expressed differently in tumours [Kibriya et al 2010].

The present study confirms that gene expression profiling based on the combination of laser microdissection of FFPE tissue and whole genome DASL assay with differential and clustering analysis is feasible. This methodology applies well to the investigation of liver disorders in which different cell sub-populations are in close relationship due to the particular liver microscopical configuration, diluting and contaminating the RNA yield if whole tissue samples are used for RNA extraction. This methodology is particularly suitable for molecular studies on hepatocellular carcinoma, in view of the characteristic morphological heterogeneity of this tumour including its mixed hepatocellular and cholangiocellular variant [Cotoi et al 2012b].

GENE EXPRESSION PROFILING

GENE EXPRESSION PROFILE OF CLASSICAL TYPE HCC COMPARED TO CHOLANGIOCELLULAR DIFFERENTIATION

As previous literature has also shown, TACE-induced changes are not only morphological. Kim et al [Kim et al 2001] described active proliferation of tumour and endothelial cells in areas adjacent to necrosis, decreasing as the distance from the necrotic margin increased. In a later study by Sergio et al [Sergio et al 2008] patients with imaging proven partial tumour ablation had a higher serum level of VEGF compared to complete responders, suggesting that incomplete TACE may induce residual tumour angiogenesis. VEGF levels also seemed to correlate with AFP before TACE, further confirming the prognostic significance of both parameters [Farinati et al 2006] and suggesting that a subgroup of HCC exists, characterized by a more aggressive biology [Sergio et al 2008]. In the same study of Sergio et al, in addition to correlating with the number of lesions, VEGF, however, revealed a particularly important correlation with survival. VEGF levels below the median were highly predictive of longer survival: Seventy percent of patients with low VEGF levels were alive after 4 years, and VEGF level emerged as the only independent predictor of survival [Sergio et al 2008].

Beta-FGF also correlated significantly with HCC vascularization and tumour size, confirming that angiogenic factors are produced and released to a greater extent in more advanced and highly vascularised disease [Sergio et al 2008]. Also, enhanced p53 expression can suppress apoptosis and stimulate proliferation in HCC after TACE [Xiao et al 2004]. Ravaioli et al observed lower expression of e-cadherin in viable tumour cells and suggested

that partial necrosis can be associated with loss of adhesion and tumour cell dislodgement in the bloodstream [Ravaioli et al 2004].

The differential analysis between the gene expression profile of the classical subpopulation of hepatocellular carcinoma and the gene expression profile of the cholangiocellular differentiation (CCd) showed 175 genes that were significantly different in expression, but without satisfying the Benjamini Hochberg Multiple Correction. A total number of 72 genes were down-regulated in HCC when compared to the gene expression profile of CCd, while 103 genes were shown to be up-regulated.

The gene expression profile data was further analysed with the FunNet Transcriptional Networks Analysis platform [www.funnet.info], an integrative tool for exploring transcriptional interactions in microarray gene expression datasets. A 5% False Discovery Rate was used to adjust the enrichment p-values for the analyzed themes. The enrichment calculations were performed by taking into account distinct levels of annotation specificity represented in the gene ontology (GO) lattice. The up-regulated genes, according to the annotated gene ontology biological processes, are more commonly involved in sensory perception of smell, response to stimulus, translation, translational initiation, regulation of translational initiation, pattern specification processes, response to heat, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, regulation of cell proliferation, regulation of insulin secretion, and cell migration. The down-regulated genes were shown to be more commonly involved in cell to cell signalling, cell adhesion, nervous system development, platelet activation, cellular protein localisation, heme transport, platelet degranulation, negative regulation of cell growth, and cell-matrix adhesion.

The genes were investigated through the Gene Sets Enrichment Analysis – gene families enquiry - (http://www.broadinstitute.org). One oncogene, four transcription factors, out of which 3 are homeodomain proteins and 2 genes belonging to the cytokines and growth factors family, were identified in the up-regulated group. In the down-regulated group, one gene for each of the following categories was found: protein kinases, tumour suppressor genes and cell differentiation markers, 7 transcription factors and 4 cytokines and growth factors encoding genes.

The differentially expressed genes were also run through the Pathway Interaction Database of the National Cancer Institute/Nature website (http://pid.nci.nih.gov/search/batch_query.shtml) for identification of the molecular pathways in which they are involved.

GENE EXPRESSION PROFILE OF HCC COMPARED TO BACKGROUND LIVER

The differential analysis between the gene expression profile of hepatocellular carcinoma and the gene expression profile of the background liver showed 77 genes that were significantly different in expression. A total number of 60 genes were down-regulated in HCC when compared to the gene expression profile the surrounding liver, while 17 genes were shown to be up-regulated.

The gene expression profile data was further analysed with the FunNet Transcriptional Networks Analysis platform [www.funnet.info]. The enrichment calculations were performed by taking into account distinct levels of annotation specificity represented in the gene ontology (GO) lattice. The up-regulated genes, according to the annotated gene ontology biological processes, are more commonly involved in negative regulation of mitotic prometaphase, response to tumour necrosis factor, response to interferon-gamma, protein modification by small protein conjugation, response to chemical stimulus, response to retinoic acid, post-Golgi mediated transport, translation and translational initiation, G-protein coupled

receptor protein signalling pathway, positive regulation of I-kappaB kinase/NF-kappaB cascade, positive regulation of apoptosis.The down-regulated genes, according to the annotated gene ontology biological processes, were shown to be more commonly involved in drug catabolic processes, hydrogen peroxide biosynthetic processes, negative regulation of peptidase activity, complement activation-lectin pathway.

The up-regulated genes were investigated through the Gene Sets Enrichment Analysis – gene families enquiry - (http://www.broadinstitute.org), one translocated cancer gene and 2 protein kinases being identified. In the down-regulated gene-group, one tumour suppressor/ transcription factor gene, one protein kinases, 2 transcription factors and 2 growth factors encoding genes were found.

The differentially expressed genes were also run through the Pathway Interaction Database of the National Cancer Institute/Nature website (http://pid.nci.nih.gov/search/batch_query.shtml) for identification of the molecular pathways in which they are involved.

Microarray technology has been applied on a large scale in order to identify the molecular and genomic mechanisms in liver carcinogenesis. In the last ten years, a great variety of gene expression profile studies have been performed.

Lau et al. [Lau et al 2000] were the first to use microarray technology to compare gene expression profiles of HCC and non-HCC liver tissues, and several similar studies have been carried out since then.

Several dysregulated genetic pathways have been identified by means of microarray analyses between HCC and adjacent non-tumoural liver. TGF-B, MAPK, IGF-2, Jak/Stat, Wnt and p53- signalling pathways were some of these altered genetic networks [Delpuech et al 2002; Okabe et al 2002; Iizuka et al 2002; Wurmbach et al 2007; Boyault et al 2007], and the most differentially expressed categories of genes were the ones involved in cell cycle progression, apoptosis, immune response, RNA splicing, DNA repair system, protein degradation, cell adhesion, metabolic enzymes, detoxification, extracellular matrix, cytoskeleton, and also cytokines, growth factors, oncogenes, tumoursupressors, and GTP-binding proteins [Delpuech et al 2002; Okabe et al 2002; Chen et al 2002; Tsai et al 2006; Zindy et al 2005; Wang et al 2009]. All these findings have been confirmed by the present study.

A very large number of genes were significantly up-regulated in HCC tissue, as the ones which regulate cell proliferation, DNA repair systems [Delpuech et al 2002; Zindy et al 2005], the contents of the extracellular matrix and cytoskeleton [Delpuech et al 2002; Tsai et al 2006], and the ones encoding secreted proteins [Wang et al 2009]. In my study, the largest group of up-regulated genes were also involved in tumour growth and proliferation and protein synthesis, according to the manual search.

Significantly down-regulated genes in HCC tissues compared to non-tumoural liver were the ones associated with immune response [Delpuech et al 2002; Chen et al 2002; Tsai et al 2006; Wang et al 2009], detoxification [Delpuech et al 2002; Iizuka et al 2002; Tsai et al 2006], encoding metabolic enzymes [Delpuech et al 2002; Okabe et al 2002; Chen et al 2002, Tsai et al 2006; Wang et al 2009], and many plasma protein coding genes [Delpuech et al 2002; Chen et al 2002; Tsai et al 2006]. My study is in accordance with the literature, most of the down-regulated genes that I found being involved in apoptosis, cell growth and proliferation, in inflammatory response and in metabolic pathways, in this order. The fact that my results are comparable to the ones in the literature, can also be extrapolated and used as an internal control for the validity of all the gene expression data that I have analysed in this work.

The microarray analysis also revealed differences in gene expression between the classical type of HCC and its CCd. Previous literature has pointed out not only morphological differences, but a genome analysis has not been performed before.

The number of genes differentially expressed shows us that there is a significant up and down-regulation of the genetic signalling. If that is a mark of aggressiveness is not still clear, and further studies are required in order to validate that hypothesis, including long term recurrence and survival studies, which I was not able to address in this work, due to the small number of cases analysed.

The pathways that seem to be down-regulated in the classical type of HCC compared to CCd are related to cell proliferation and survival, with most of the genes that were manually searched and characterised playing a role in cell cycle progression and apoptosis.

One of the disadvantages of these assays, as the current study has also shown, is that many individual genes, that were found to have different expression in HCC when compared to the background liver, cannot be confirmed form a microarray study to the other. Therefore, it seems more useful to concentrate on the above mentioned categories of genes.

It has also been suggested that FFPE tissue instead of FF tissue can be successfully used in microarrays for identifying groups of genes that may be expressed differently in tumours, but not single genes, because of the variance in the number and content of the list of genes detected [Kibriya et al 2010].

Another disadvantage of the techniques uses in my study is that the measurement of gene expression in the WG-DASL assay was done indirectly and it depended on the "labelling competition" in the PCR amplification. Thus, changes in hybridization signal might have not reflected changes of the number of transcripts in the most accurate way [Bibikova et al 2004a]. Also, the gene expression was examined only at the mRNA level, which may not be equivalent to protein expression. This opens the door for future studies in order to validate the results.

The genomic signatures of HCC can still be used to help characterize the molecular changes responsible for HCC development and it could be extremely valuable in the development of new tumour markers. Furthermore, novel therapeutic targets can be identified through the means of WG-DASL assays, even when used in combination with laser microdissection and RNA extraction from paraffin embedded tissue.

CONCLUSIONS

The histological evaluation of the post-TACE tumours showed that tumour destruction was achieved in 42.85% of the patients, with complete destruction in 25% of them, broadly replicating what is described in the literature.

Most of the post-TACE viable tumours were moderately differentiated, but there were also biphasic tumours, as a mixture of moderately differentiated with well or poorly differentiated tumours. Fourteen percent of them showed a mixed hepato-cholangiocellular phenotype, observation which supports the idea of a phenotypical differentiation associated with TACE.

In this study I presented a novel approach to gene expression profiling based on the combination of laser microdissection of FFPE tissue and whole genome DASL assay. I was able to obtain gene expression signals from hepatocellular carcinoma, as well as from cirrhotic and non-cirrhotic liver background. The whole genome DASL assay can be used on FFPE samples obtained by laser microdissection, despite RNA degradation and chemical

modification, giving the opportunity to investigate specific cell populations from archival histological material. Thus, differential and clustering analysis was feasible for investigating the gene expression profiles obtained.

The statistical results showed that the largest groups of up-regulated genes in HCC when compared to the background liver are involved in tumour growth and proliferation, and protein synthesis. The groups of down-regulated genes are related to apoptosis, cell growth and proliferation, inflammatory response and metabolic pathways. These results are similar to the ones found in the literature.

The results also showed a difference in the gene expression profile of the classical type of HCC when compared to the cholangiocellular differention of the tumour after TACE, that seems to be related but not limited to to cell survival and invasive potential. These results just open the door for future studies, but add an important piece of information to a field that is yet to be discovered.

New differential and clustering analysis between the subtypes of HCC (classical and cholangiocellular differentiated HCC) and between the HCC arising in different aetiological contexts are to be performed in the future, in order to get a better inside view on the pathogenesis of liver cancer.

Further validation of the study is necessary by means of immunohistochemistry and RT-PCR, in the attempt to identify single genes that may be targeted in hepatocellular carcinoma therapy or that might explain the various morphological aspects of HCC after TACE, that might be the key to a better treatment of malignancy.

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