Comparative Genomic Classification of Human Hepatocellular Carcinoma

Doctoral thesis

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Budapest
2008

1. Introduction

Human hepatocellular carcinoma is the fifth most common human malignancy world-wide, and it is responsible over 500,000 deaths annually. The etiological background of HCC is relatively well characterized. Most of the cases develop on the background of continuous liver injury and regeneration cycles and could be associated with chronic hepatitis B or hepatitis C virus infections as well as with other toxic or metabolic disturbances resulting in liver cirrhosis. Hepatocarcinogenesis is generally considered to be a multi-step process progressing through consecutive stages of pre-neoplastic and neoplastic alterations. Although it is obvious, that genetic or epigenetic inactivation of multiple regulatory genes are required for HCC formation, unfortunately, the molecular details of this process are not fully understood. Loss of heterozygosity (LOH) and epigenetic inactivation of tumors suppressor such as Rb, E-cadherin, p16 can be already detected in early neoplastic lesions. Meanwhile, advanced HCCs are characterized by large scale genomic alterations among which point mutations of p53 and β-catenin, as well as amplifications of the 1q and 8q and deletions of the 8p, 17q etc. chromosome arms are the most frequently encountered. Therapeutic intervention in HCC is limited to surgical resection or liver transplantation in early stage patients. Currently, there is no cure available in the advanced stages as hepatic tumor cells are notoriously resistant of almost all chemotherapeutic agents. Fortunately, a new generation of anti-caner drugs is under development. These target specific pathways of tumor progression and some of them such as bevacizumab, bortezomib or sorafenib achieved encouraging response rates in the initial phases of clinical testing. Therefore, to overcome the difficulties caused by diverse pathogenetic background of the liver carcinomas, new molecular classification methods are needed to identify patients who would benefit the most from the targeted therapeutics.

Introduction of novel high-throughput techniques resulted in dramatic increase of the available genomic information. Simultaneous detection of the global cancer transcriptome became a reality. Expression microarrays have been successfully applied to the characterization of multiple human malignancies including human HCC. Expression profiles associated with various clinical and pathologic variables such as viral agents, presence of cirrhosis etc. have been extracted. Yet, complexity of human tumor data often prevents direct identification of molecular signatures marking the activation of a specific oncogenic pathway. However, these signatures can be revealed in vitro and in vivo models, where the number of experimental variables can be rigorously controlled. Comparative functional genomics, through alignment of expression profiles from evolutionally conserved orthologus genes,

allows detection of the pre-defined transcription signatures in human tumors. Thus, diversification of human neoplasms based on their molecular background can facilitate development of novel classification systems. in the archived pathologic specimens.

During our investigations we used global gene expression profiling to identify the c-Met regulated expression signature in mouse primary hepatocytes. Analysis of microarray data reveled several new c-Met target genes. A number of these genes were involved in oxidative stress response indicating for the first time that HGF/c-Met signaling plays a significant role in regulating hepatocyte homeostasis. Assessment of the c-Met induced expression signature genes in human hepatocellular carcinomas identified a subgroup of HCC patients with probable c-Met activation. Presence of the c-Met activated expression pattern was associated with aggressive phenotypic traits and resulted in shortened survival of the affected patients.

The biologic significance of the c-Met induced expression signature is confounded by numerous observations. Presence of the c-Met signature showed significant correlation with increased micro vessel density, and high vascular invasion rates. These findings are in agreement with previous reports that designated c-Met as a dominant promoter of tumor angiogenesis and metastatic spread. In addition, prevalence of the c-Met signature in human HCC patients had significant effect on disease prognosis. This was also evidenced by the clear separation of the c-Met positive tumors with six different prediction algorithms. The prediction model built from evolutionally conserved c-Met targets demonstrated that significantly lower survival rate among patients with predicted c-Met activation than in c-Met negative controls. The optimal c-Met classifier consisted of genes whose function is related to cell adhesion, organization of the actin cytoskeleton and diapedesis, indicating that the c-Met induced transcriptional program is directly involved in metastatic transformation of the tumor cells. Interestingly, the metastatic colon and ovarian tumors also showed equivocal activation of the hepatocyte derived c-Met expression signature. This suggests that signaling mechanisms regulating vascular invasion and metastasis formation considerably overlap between malignancies regardless of cellular origin.

We identified the HGF induced expression signature in c-Met conditional knock-out primary hepatocytes. This model allowed detection of c-Met activated genes in fully differentiated cells. Moreover, interference from functionally related pathways such as the autocrine stimulation of the TGFα/EGFR signaling could be completely excluded. Nevertheless, it is probable that in human tumors the c-Met activated expression pattern can be induced by alterations in multiple signaling pathways. Comparison of human HCC with tumors arising transgenic mice, found significant overlap between the Myc/TGFα and the bad

prognosis associated expression profiles. Although, direct comparison of the expression signatures of the different tyrosine kinase pathways has not been performed, we suppose that the commonly observed phenotypic similarities are also reflected in the transcriptional programs.

Functional classification of the significant genes can provide mechanistic explanation of the functional alterations observed in the c-Met knock-out cells. Absence of the HGF induced cell cycle, cytoskelatal and cell adhesion genes corresponds with the impaired motility and decreased proliferation potential of the knock-out hepatocytes. On the other hand, over-expression of the oxidative and xenobiotic stress-response genes suggests disturbance in the metabolic homeostasis of these cells. Simultaneous induction of the NRF2 transcription factor and its target genes (EPHX1, ALDH1A1, ALDH1A7, GSTA3, GCLC etc) indicates of profound alteration of glutathione metabolism. Defective activation of ERK signaling and a concomitant shift towards an RXRα and PPARγ dominated transcriptional program is one of the possible confounding factors behind the metabolic changes. Thus, our results for the fist time, provide unequivocal evidence that HGF/c-Met signaling plays an essential role in maintaining hepatocyte homeostasis.

Recently, new methodologies became available to extract RNA from paraffin-embedded samples, which previously were not considered a reliable source of RNA recovery. According to earlier studies, replacement of formalin with non cross binding fixatives can reduce fragmentation of nucleic acids and therefore, improves PCR amplification rates. The ability to extract the genomic information from the paraffin embedded specimens has a overwhelming significance as it could open up invaluable pathology archives for molecular research. Moreover, reliable transcriptomic analysis of the routinely processed pathologic specimens could facilitate the implementation of novel molecular diagnostic tests into the clinical practice. Formalin is used for routine processing of pathology specimens because of its excellent preservation of morphologic details. Unfortunately, formalin is considered an inadequate fixative for molecular studies, as it forms covalent cross-links between peptide groups and nucleic acids. Pathology archives filled with thousands of well documented, uniformly handled tissue specimens are treasure chests of retrospective studies. Thus, an ideal fixative, which equally capable to preserve structural details and molecular information, has long been pursued by many researchers. Organic chemicals such as ethanol, methanol, and acetone have been found to provide better DNA amplification rate in PCR than formalin. According to other studies RNAlater, a newly introduced fixative, also provided superior RNA preservation than formalin. By using improved RNA isolation techniques, amplification

of viral transcripts was possible form over 20-year-old paraffin embedded samples. Successful messenger RNA recovery from formalin fixed paraffin-embedded specimens has also been reported, although amplification rate greatly varied according to storage and fixation times. While some authors found that longer, up to 1 week fixation increased RNA recovery, others claim that higher yields can be achieved after 3 to 6 hours formalin treatment. Our results demonstrated that no significant difference could be demonstrated between the RNA amplification rates of the formalin, RNAlater or acetone fixed endometrial samples. While formalin fixed specimens showed better preservation of tissue morphology and more uniform immunohistochemical stains. Although some of these findings are not in full agreement with past publications which described no differences in the integrity of microscopic structures or reactivity with immune stains following RNAlater fixation. We also discovered that above 225 bp the PCR yield gradually decreased with the increasing product length. This is consistent with expectation that longer amplicons are more sensitive for RNA fragmentation, caused by covalent cross links and heat exposure during the embedding. We also have to note that larger specimens where formalin penetration time is extended can have dramatically different RNA preservation characteristics. Nevertheless, new tissue processing protocols could significantly enhance our access to the genomic information hidden.

Expression studies are also often hindered by the small amount of available tissue material. Over the times, various template and signal amplification methods have been devised to overcome this problem. The progress in microarray design and manufacturing means that oligonucleotid arrays replaced cDNA arrays as the dominant platform. While, indirect, aminoallyl conversion based coupling became the standard template labeling method. To achieve better signal intensity and reproducibility with the new types of arrays, modification of the conventional linear RNA amplification protocols has also become necessary. The RNA amount obtained from microdissected or biopsy samples is often not sufficient for microarray analysis. Successful hybridization of long oligonucleotide arrays requires over 100 ng poly(A) or at least 20 μg total RNA. Fluorescently labeled anti-sense strand RNA targets can be generated from conventional T7 RNA polymerase-based amplification reactions. However, the amplification reaction developed by us result in sense-strand aRNA product, which can be further processed into amioallyl modified cDNA template. This method provides better signal intensity and more balanced labeling with the two fluorescent dyes. Assessment of data obtained with the co-hybridization of normal and Myc/TGFα transgenic tumor sample demonstrated excellent correlation between the repeated T7T3 amplification reactions. While other methods also achieved good reproducibility, only a

handful of studies investigated preservation of original expression profiles following RNA amplification. We also compared expression ratios of significantly regulated genes form the total and the corresponding amplified RNA samples. Clustering of Myc and Myc/TGFα tumor samples with and without amplification confirmed that genotype specific expression patterns are retained. Zhao et al. found similar average correlation (0.74-0.86) between breast tumor microarrays hybridized with poly(A)RNA or single round T7 aRNA. Reproducibility remained acceptable even when T3N9 amplification was applied alone through multiple rounds. Through the analysis of transgenic mouse tumors we also validated the performance of the T7T3 method on laser microdissected samples. However, these data displayed persistent bias in a minor fraction of gene expression ratios introduced by either the microdissection or the amplification steps.

Our results therefore demonstrate that careful experimental design in combination with state-of-the-art laboratory methods can open up archived and limited availability pathologic samples for global expression profiling. Meanwhile, identification of pathway-specific expression signatures in tightly controlled experimental models can significantly contribute to the discovery of novel intracellular regulatory mechanisms. In combination with a comparative functional genomics analysis of the cancer transcriptome, these methods will result in molecular classification schemes essential for individualized cancer therapeutics.

2. Aims

We aimed to investigate the following questions:

1. We wanted to **test different tissue fixation methods to improve nucleic acid recovery from archived, paraffin embedded pathologic specimens**. We assessed preservation of the morphologic features and reactivity with the immunhisotchemical stains with each fixative.

2. We aimed to **develop a novel RNA amplification method** where the aRNA product possesses **optimal labeling and hybridization characteristics on the long oligonucleotide microarrays**, and it preserves the original genomic information with high fidelity.

3. We wanted to **identify the global effect of recombinant HGF treatment on the hepatocyte transcriptome**. We also wanted to determine the dominant differentially regulated gene categories responsible for HGF evoked phenotypic changes.

4. By utilizing a comparative functional genomic approach we **investigated presence of c-Met induced transcription signature in human HCC** and its association with the standard clinico-pathologic variables.

3. Methods

Collection of human endometrial and liver samples

Human endometrial tissue samples were collected from eighteen hysterectomy specimens immediately after surgical removal, and were placed into one of the three tested fixatives (acetone, formalin, RNAlater). Tissues were fixed in either in 5 mL formalin for 24 hours on room temperature or in 5 mL CuSO4 saturated acetone for 15 to 60 minutes. RNAlater fixed tissues were kept in the fixative for 24 hours on room temperature than washed with PBS. All samples were processed following routine embedding procedures.

Hundred and thirty nine human hepatocellular carcinoma samples were collected from patients who underwent surgical lobectomy or orthotopic liver transplantation in various institutions. In 70 cases matching non-neoplastic liver samples were also available. Hybridization control was generated from pooled normal total was isolated from 18 patients who underwent surgery following traumatic liver injury. All sample collection and storage was approved by the ethical committees of all participating institutions.

Mouse models

The c-Met hepatocyte specific conditional knock-out mouse model was generated in the Laboratory of Experimental Carcinogenesis as it has been previously described. Briefly, exon 16 of the c-Met receptor gene was flanked by Cre/lox recognitions sites by homolgous recombination in mouse embryonic cells. Chimeric offspirngs were inbred through multiple genotypes to generate c-Met$^{fl/fl}$ mice. Crossing these mice with an Alb-Cre$^{+/-}$ strain resulted in the c-Met$^{fl/fl}$/Alb-Cre$^{+/-}$ conditional knock-out genotype. As expression of the Cre-recombinase was driven by the human albumin promoter the deletion was specific for the fully differentiated hepatocytes.

Detailed characterization of the c-Myc single and c-Myc/Tgfα double transgenic mice is available in previous publications. Tumor samples were collected from eight month old animals. Livers from wild-type B6CBA/F1 mice were used as normal control. All animal experiments were approved by National Institutes of Health Animal Welfare Committee and the experiments were conducted according to current NIH guidelines.

Primary hepatocyte isolation and culture

Primary mouse hepatocytes were isolated form both c-Met conditional knock-out and from Cre control animals with a two step collagenase perfusion. Viable hepatocytes were

separated from the non-epithelial cell component by Percoll-gradient centrifugation. Two million hepatocytes were plated on collagen coated culture plates. After four hours initial attachment the plating medium was replaced by serum free medium. Next morning recombinant human HGF was added to the cell in 50 ng/ml concentration. The control and c-Met primary hepatocytes were washed in PBS and collected in 5 ml Trizol reagent in triplicates after for half, two, twelve and twenty four hours of treatment. Non-treated, plated control cells were used as hybridization control.

Laser microdissection

Six micron thick frozen sections were cut from three well-developed mouse liver tumors from each investigated genotype. Freshly cut sections were immediately stained with alcian blue dye, followed by quick dehydration in ascending row of ethanol dilutions and xylen. Air dried sections then were microdissected with the Arcturus PixCell IIe lase capture microdissection system. From each a sample approximately 3000-4000 cells were collected into a guadinium thiocyanate RNA lysis buffer.

RNA isolation

Total RNA from the laser microdissected samples was isolated by the Qiagen RNA Micro kit with an on-column DNase digestion following the manufacturer instructions. The primary mouse hepatocytes were lyzed in the culture dishes in Trizol reagent and total RNA was purified according to the standard protocol. For RNA extraction from the human and mouse tissues we utilized the CsCl isodensity centrifugation method. From the archived endometrial samples five 10 micron thick sections were cut. Following de-paraffiniztion total RNA was extracted with the High Pure Paraffin kit. RNA concentrations were determined with UV spectrophotometry. The integrity of the samples was assessed with the Agilent Bioanalyzier 2100 electrophoresis system.

Quantitative real-time PCR

Real-time PCR quantification of the β-globin and GAPDH transcripts in the paraffin-embedded endometrial samples was performed with different length products. C_T values obtained with the SYBR Green fluorescent reporter were recorded in triplicate reactions. Relative transcript abundance between the different samples was calculated with the REST algorithm. Product specificities was verified with a melting curve analysis and agarose gel electrophoresis. Validation of microarray results was accomplished by quantitative PCR assessment of ten randomly selected differentially expressed genes.

Microarray platforms

The Compugen mouse oligonucleotide library contained 21997 65 bp probes representing 19140 unique transcripts. The Operon Human V2 oligonucleotid library consisted of 21329 70-mer probes. Both libraries were printed to amino-silane coated glass slides in a 160 micron probe density by the NCI Advanced Technology Center.

Probe preparation and microarray hybridization

Total RNA samples were reverse-transcribed to aminoallyl-UTP modified cDNA. Labeling of the samples with the Cy3 and Cy5 fluorescent dyes took place following activation of the aminoallyl chains on a high pH. The purified, fluorescently labeled templates were hybridized to long-oligonucleotid microarrays against the same mouse or human reference. Duplicate hybridizations were performed in a reverse-flour design. Arrays were scanned with a GenePix 2000A scanner. After fitting the localization grid, individual probe intensities were determined with the GenPix software.

Statistical analysis

Filtration and normalization of raw signal intensity data was completed in the NCI MadB website. Initial unsupervised classification of tumor samples was based on the hierarchical cluster analysis with Michael Eisen's Cluster and TreeView software. Differentially expressed gene sets were identified in a two sample t-test, performing 1000 random permutations of data labels to estimate the false discovery rate. A genomic prediction model was built using six different prediction algorithm available in the BRB-Array Tools software package. Survival statistics were calculated in R (http://www.R-project.org/).

4. Results

4.1 Effect of different fixatives on RNA preservation in paraffin embedded endometrial samples.

We tested the effectiveness of RNA preservation with three tissue fixatives (formalin, acetone and RNA-later) on routinely processed, paraffin embedded endometrial samples using quantitative real-time PCR analysis. No difference was observed in the amplification rate of the different size GAPDH and β-globin products between the alternative fixation methods. Below 225 bp product length the amplification efficiency remained acceptable with all fixatives, but it rapidly decreased with longer amplicons. In addition, formalin fixation provided the best morphology on H&E sections, and showed the most uniform reactivity with the claudin 4 and 7 antibodies. On the other hand, in RNA-later fixed samples both the pattern and the intensity of the immunohystochemical stains were found to be uneven.

4.2 Development of a T7T3 based linear RNA amplification protocol for generating a sense strand product.

We developed a two-round linear RNA amplification protocol to generate sense strand aRNA product. By combining oligo(d)T20-T7 amplification in the first, and random nonamer (N9)-T3 based amplification steps in the second round we achieved approximately 2 x 104-fold amplification rate of the starting messenger RNA. The average length of the amplified fragments was ranging between 150 and 1,350 bp. The aRNA products amplified either from frozen or laser microdissected samples performed well in the subsequent aminoallyl cDNA labeling steps, and provided good signal intensities when hybridized to oligonucleotide microarrays.

4.3 Evaluation of reproducibility of linear RNA amplification on microdissected transgenic mouse tumors.

Reliability of the T7T3 amplification method was further evaluated by microarray analysis of Myc and Myc-Tgfα transgenic mouse hepatocellular carcinomas. Comparison of the at least 2-fold regulated gene expression ratios genes showed good reproducibility (Pearson r=0.9) between the repeated amplification reactions. When compared to non-amplified RNA samples the number of significantly regulated genes was similar (1135 vs. 1136) following T7T3 amplification. Hierarchical cluster analysis confirmed the high-grade similarity (r=0.79) and

preservation of genotype specific expression patterns between the amplified and non-amplified samples.

4.4 Identification of 730 HGF/c-Met target genes in c-Met conditional knock-out mouse hepatocytes.

We identified the set of hepatocyte expressed c-Met specific target genes. Comparison of expression profiles, obtained from control and c-Met conditional knock-out mouse primary hepatocytes following recombinant HGF treatment at multiple timepoints (30 min, 2, 12 and 24 hours) as well as from non-treated serum starved cells (0 hours), detected 690 significant genes with HGF/c-Met responsive transcriptional patterns. In addition 67 genes showed sustained expression differences between control and c-Met knock-out samples in all comparisons. These potentially represent long-term transcriptional adaptation of the knock-out cells.

4.5 Functional analysis of the HGF induced gene set implicates c-Met as a key regulator of cell motility, proliferation and oxidative homeostasis.

We performed functional analysis of the HGF induced genes utilizing Gene Ontology annotations and pathway recognition tools. In addition to the immediate early response genes (Hmga1, Egr1, JunB, MafF) induced at 30 minutes and 2 hours, cell adhesion, cell motility and cytoskeletal genes (Fn1, Neo1, Robo1, Cldn2, Arpc1b, Cap1, Nck2, Msn etc.) constituted the most significant c-Met regulated categories. Interestingly, expression of multiple oxidative stress response genes including the transcription factor Nrf2 and several of its targets were suppressed by HGF treatment. For the first time, these findings implicate HGF/c-Met pathway as an important regulator of cellular homeostasis.

4.6 Comparative genomic analysis of human HCC samples identifies a subgroup of tumors with prominent c-Met activated expression pattern.

Using a comparative functional genomic approach we assessed activation of the HGF/c-Met pathway in human hepatocellular carcinomas. Our analysis revealed, that a subset (67/242, 27%) of hepatocellular carcinomas displayed similar transcriptional pattern of the c-Met target genes to the HGF treated primary mouse hepatocytes, suggesting active c-Met signaling. The same c-Met positive group could also be detected in HCC and metastatic colon cancer data sets published by an independent groups.

4.7 Presence of c-Met signature in human HCC is associated with increased MVD, micro-vascular invasion rate and poor prognosis.

From the different clinical and pathological variables, presence of the c-Met activation signature in human HCC showed significant positive correlation with increased microvessel density (90.8 ± 6.7 vs. 44.5 ± 6.2, $p<0.001$) and microvascular invasion rate ($\chi2=4.0$, $p<0.05$). Patients with c-Met signature positive tumors also had significantly (log-rank test $p<0.05$) shorter median survival (35.1 ± 7.15 months) than other patients (70.3 ± 9.7 months) in the data set.

4.8 A genomic model based on the c-Met signature is able to predict survival of HCC patients with 83-95% accuracy.

We developed a genomic model to predict disease outcome in hepatocellular carcinoma patients based on their c-Met activation status. An optimal classifier, composed of 111 c-Met target genes, was identified in a 60 sample (30 c-Met positive and 30 c-Met negative) training set with six different prediction algorithms (NN1, NN3, SVM, CCP) following a leave-one-out cross validation strategy. When applied to the 79 sample validation set. The above prediction model was able to diversify hepatocellular carcinoma patients into good and poor prognosis groups with 83-95% accuracy.

5. Conclusions

There was no difference in the integrity of RNA obtained from the formalin, acetone or RNAlater fixed specimens. Short, less than 225 bp products could be efficiently amplified from archived tissues. Consistency of immunohistochemical stains was superior with formalin than with the other two fixatives.

The T7T3 method achieves on average 2×10^4 linear RNA amplification rate, while the average product length ranges between 150 and 1,350 bp. The aRNA product from the T7T3 amplification could be reversed transcribed into anti-sense strand, aminoallyl modified cDNA.

Microarray profiles of T7T3 amplified samples show strong correlation with non-amplified controls (Pearson r=0.9) as well as with experimental replicates (Pearson r=0.78). The linear RNA amplification preserves the genotype specific expression patterns of the transgenic mouse liver tumors.

Activation of the HGF/c-Met signal transduction pathway in mouse primary hepatocytes, in addition to the activation of immediate early response genes, induces expression of genes involved in cell motility, adhesion and cytoskeleta organization. The c-Met pathway controls transcriptional activation of oxidative stress response genes. Adaptation of c-Met knock-out hepatocytes permanently alters expression levels of 67 genes.

Presence of the HGF induced expression signature suggests activation of c-Met signaling in a set of primary human liver tumors and metastases. In these lesions microvessel density (90.8 ± 6.7 vs. 44.5 ± 6.2, $p<0.001$) and microvascular invasion rate ($\chi^2=4.0$, $p<0.05$) are significantly higher than in other tumors.

A genomic prediction model constructed from the HGF/c-Met signature genes predicts prognosis of HCC patients with 83-95% accuracy. The average survival with c-Met signature positive tumors is significantly shorter (35.1 ± 7.15 months) than the rest of the HCC patient’s (70.3 ± 9.7 months).

6. List of Original Publications

7.1 List of authored publications used for the dissertation.

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