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MOLECULAR EVENTS IN HEPATIC PRENEOPLASIA: A REVIEW

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PRENEOPLASTIC LESIONS IN HUMAN LIVERS

Human studies have been done which indicate that dysplastic nodules between 1 mm and 1 cm in diameter are precancerous (International consensus Group for Hepatocellular Neoplasms 2009) Microscopic foci less than 1 mm in diameter are referred to as dysplastic foci. Large cell change (previously called large cell dysplasia) is associated with hepatocellular carcinoma (HCC) but does not represent a true precursor lesion for HCC. On the other hand small cell change (previously called small cell dysplasia) is considered a direct precursor of HCC (Desmet 2009). So far, changes in molecular pathology of dysplastic foci have not been established.

MOLECULAR PATHOLOGY OF DYSPLASTIC NODULES

Molecular changes in gene expression, have been reported comparing normal liver (NL), cirrhotic liver (CL), low and high grade dysplasia nodules (LGDN and HGDN) and early HCC (EHCC). There was difficulty, however, in distinguishing high grade dysplastic nodules (HGDN) from early HCC (EHCC) (International Consensus Group for Hepatocellular Neoplasms 2009). Three histochemical markers of changed gene expression, which indicate malignant transformation have been identified e.g. heat shock protein 70 (HSP70) (Kojiro, 2005), glutamine synthetase (GS) (Roncalli et al, 2007) and glypican 3 (GPC3) (Kojiro, 2006). GPC3 however, may stain positive in regenerating hepatocytes in hepatitis C (HCV) (Abdul-A, et al, 2008). When these 3 stains were applied to resected small liver lesions, the expression of two of the 3 positive markers had a sensitivity of 72% and a specificity of 100% establishing HCC (DiTommaso et al, 2007).

In a study comparing hypermethylation of CpG islands in cirrhosis, HCC (CL) and dysplastic nodules (DN) (LGDN and HGDN), several genes were found to be hypermethylated in HCC (APC: 82%, GSTP, 77%), RASSF1A 16%, p16: 48%, COX-2:35%/and E-cadherin: 35%. In comparison, APC, E-cadherin and GSTP1 were hypermethylated in APC. COX-2, p16 and RASSF1A were hypermethylated in DNs. TIMP-3 was only hypermethylated in HCC. These results indicated an increase in frequency of hypermethylated genes in a stepwise manner in methylated CPG islands during multistep hepatocarcinogenesis (Lee et al, 2003). These results provide evidence of epigenetic events in gene expression from CL to DN to HCC. Aberrant epigenetic events are primary events which affect multiple genes and cellular pathways in a non-random fashion causing heritable

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changes in gene expression, which create a predisposition to the development of preneoplastic change and progression to cancer formation (Sawan et al., 2008).

Microarray analysis of livers has been done to study the progression of preneoplastic lesions, including low-grade dysplastic nodules (LGDNs) and high-grade dysplastic nodules (HGDNs) to the advanced stage of HCC (Nan et al., 2005). A clinical series of hepatocellular nodular lesions from 42 patients was studied using 18,000 unique gene probes, (7 LGDNs, 7 HGNS, 9 G1 HCCs, 10 G2 HCCs, and 8 G3 HCCs). Hierarchical cluster analyses were performed of expression profiles on nodular lesions including 10,376 genes. Two clusters were defined (Cluster #1: LGDNs and HGDNs), (Cluster #2: G2 and 3 HCCs) and most G1 HCCs. These findings showed reproducible large scale changes in gene expression, which distinguished preneoplastic lesions from HCCs as well as different grades of HCC. Three of the 9 G1 HCCs showed an expression profile with greater similarity to the preneoplastic nodules. This suggests that G1 HCCs are molecularly heterogeneous, bordering on a transition from premalignant to overt HCCs at the molecular level. Adjacent non-neoplastic liver expression profiles were clustered apart from the dysplastic nodules, indicating that dysplastic nodules differed molecularly from normal liver.

The question is: which genes are involved in the progression from LGDNs to HGDNs to HCC G1-2-3? Gene selection from LGDNs to HGDNs to HCC G1-2-3 was done to statistically identify which genes changed their expression from one class (grade) to the next. The gene selection that showed class expression spikes were identified using 3,084 probes with expected maximum occurrence of false discovery of 63 genes. Hierarchical clustering of these genes showed that the predominant grade-associated expression profiles are those with either positive or negative correlations with grade at the $p=0.001$ level. Not surprisingly, cluster 1 cell cycle functions clustered with HCC grade 3 including DNA replication, chromatin remodeling and cell proliferation. Cluster 2 included genes which showed a gradual increase in expression from preneoplastic lesions to G2 HCC with a sharp increase in G3 HCC. These genes included those involved in protein synthesis including ribosomal proteins, translation initiation, elongation factors and constituents of the spliceosomes. The genes found in Cluster 3 were characterized by a gradual but large-magnitude decline in transcript levels from LGDN to 3 HCC. They play central roles in primary liver function which implies good differentiation. These genes involve fatty acid and lipid metabolism, detoxification pathways and synthesis of complement and coagulation factors. The progressive loss of function occurs as progression occurs from LGDN to G3HCC. 24 genes that contribute to increasing malignant behavior in cluster 1 included oncogenic transformation or tumor suppression genes. Growth factors, apoptotic genes and cell adhesion molecules were also cluster 1.

What are the genes expressed that accurately classify the stages of tumor progression from LGDNs to G3 HCC? Thirty to 50 genes per grade was optimal. Twenty up regulated and 20 down regulated genes were identified per grade. One hundred early stage (LGDNs, HGDNs and G1HCC) and late stage (G1, 2 and 3 HCC) genes were studied at a 91% classification accuracy. For the early stage samples, 100% of the specimens were correctly classified. For late stage samples all but two arrays were classified correctly. In summary, there was a clear separation of dysplastic nodules from overt carcinoma but also grade-specific sub clusters of HCC and ND using unsupervised hierarchical clustering analysis. There was a clear difference in molecular signature between histological grades except that there was some molecular heterogeneity in G1 HCC. Three G1HCCs were grouped into precancerous nodules (Nan et al., 2005).

Several biomarker genes have been used to detect early liver cell neoplasms. They include HSP70, GPC3, TERT, STK15 and PLA2 (Wumbach et al., 2006). The combination of a 3-

gene signature (GPC3, XLKDI, and BIRC5) has been used to distinguish dysplastic nodules from early HCC (Llovet et al., 2006). The search for new and more accurate markers to discriminate dysplastic nodules from small HCC was done using microarray analysis on 75 samples (48 patients) (Llove et al., 2006). Thirteen samples from cirrhotic tissue, 17 dysplastic nodules and 35 HCCs were studied. The results were compared with samples of normal liver tissue from 10 healthy patients undergoing liver tissue removal. The samples were morphologically classified according to the International Working Party. Two main clusters formed; one included 34/35 HCC samples; the second included all 30 preneoplastic conditions (cirrhosis and dysplastic nodules). The preneoplastic cluster was split into cirrhotic and dysplastic sub clusters. The expression of 8 genes was up regulated by cirrhosis. The expression of 24 genes differed when cirrhosis was compared with dysplasia. The expression of 93 genes differed when dysplasia was compared with early HCC. The expression of 53 genes differed when dysplasia was compared with HCC. Eight gene expression changes were confirmed by PCR, one gene for cirrhosis (CLDN 10), 3 for dysplasia (GREM Z, EPO and NRG1) and 4 for HCC (ASPM, PRIMI, HMMR and IRAK1). These changes in gene expression define a spectrum of molecular markers for different stages of hepatocellular neoplastic progression. Cirrhosis showed marked up regulation of JAG1, a ligand of the Notch receptor and members of the Toll pathway (SATI, CXC19-11, and IFN γ inducible genes). Dysplasia showed up regulation of genes involved in cytokine-cytokine receptor and the Jak/STAT pathways (EPO, EPOR, C15H, STAT3 and SOCS3) as well as cell cycle regulation (GADD 45B, GADD 45G and GADD45A). Early HCC showed changes in all 45 pathways including down regulation of several members of the Toll-like receptor pathway (IFNAR1, TLR4, and CD14) and up regulation of the wnt pathway as well as PTCH, a member of the Hedgehog pathway. It was concluded that several components of signaling pathways in the preneoplastic stages were dysregulated and further altered during transition to HCC, especially the Jak/STAT signaling pathway (Llovet et al., 2006).

The role played by wnt/ β -catenin signaling in preneoplastic nodule/HCC progression was examined. The most frequently mutated oncogene in human HCC is CT-NMB1 encoding β -catenin protein (Metodieva, 2008). In the absence of wnt signaling, β -catenin is phosphorylated by functional interactions with glycogen synthase (GSK)-3, AXIN, AXIN2 and the APC protein, leading to β -catenin degradation by the ubiquitin-proteasome system (UPS). The activation of the wnt signal induces β -catenin stabilization through inhibition of GSK-3 β activity and the transfer of β -catenin into the nucleus where β catenin activates the expression of cmyc, cyclin D1, WISP, fibronectin, MMP, survivin, glutamine synthetase, CYP2E1, CYP1a2 and glutathione S transferase (Metodieva, 2008; Cieply et al., 2009). Frizzled (FZD), which codes for a transmembrane receptor in the wnt signaling cascade, releases β -catenin from E-cadherin, releasing established cell-cell adhesions (Villanueva et al., 2007). These mutations create oncogenes in the wnt signaling pathway in HCC. Truncated forms of β -catenin are also found in some adenomas (Chen et al., 2002). A subgroup of adenomas with atypical features associated with HCC, (either borderline lesions or associated with HCC) showed activation of β -catenin (Monga, 2006). This subgroup of adenomas has a higher risk of HCC. β -catenin activation has been reported in various stages of tumorigenesis including dysplastic foci (Monga, 2006). β -catenin activation is also found in oval cells (liver stem cells) which might be precursors of a subset of HCCs (Apte, et al., 2008). β -catenin activation mediates complex chromatin dynamics. CBP/p300 (Histone acetyltransferase) (HAT) interacts with β -catenin, leading to activation of many β -catenin target genes (Mosimann, et al., 2009). Large chromatin regions are histone acetylated as a consequence. β -catenin can localize histone-repositioning machineries to its target gene loci. β -catenin interacts with histone methyltransferase SET, which methylates H3K4 (me1, me2 and me3) to activate transcription sites through efficient RNA polymerase II-mediated transcription.

When the gene expression of early HCCs was compared with premalignant lesions using an oligonucleotide array of 12,600 genes in 7 cases of nodule within nodules, a set of 95 genes distinguished the HCC and non-HCC. Liver HSP 70 was the most up regulated, confirmed by RTPCR and immunohistochemistry. Cyclase-associated protein 2 (CAP2) was up regulated and immunohistochemistry was positive in the HCCs, but only focally positive in the preneoplastic nodules (Sakamoto et al., 2009).

WHY DO DYSPLASTIC NODULES AND HCC PREDOMINANTLY OCCUR WHEN CIRRHOSIS DEVELOPS?

Liver cell regeneration is inhibited when cirrhosis develops primarily because of shortening of telomeres and a growth inhibiting environment, which selects for proliferating cells (Kojiro et al., 2005). Of course other factors are involved including the changes in the expression of genes such as p53, ARF, MDM2, gankyrin, p27, TGF β , IGF2R, Rb and p16. Also what could occur is cell cycle arrest/apoptosis in response to oncogene induced stress, oncogene-induced DNA damage, chromosome breakage and oxidative damage. Oncogenic pathways include myc, p13K/AKT and pten. Developmental pathways include prickly 1, wnt β -catenin, hedgehog and met (Farazi et al., 2003). These factors converge on cell proliferation, stem cells, cellular immortality and reversal of telomerase shortening (Thoreirson 2006). Regulation of these phenomena by epigenetic mechanisms is major in establishing immortality of the cellular changes that develop. MicroRNA expression regulates these changes that progress to the development of HCC. miR-21, 34a, 221/222 and 224 are increased and miR-122, 145, 199a, 106a, 92, 17.5p, 20 and 18 are decreased. Adenomas and dysplastic nodules do not show changes in miR-21, 224, 106a, 203, 106, 222, 122a and 422b (Braconi et al., 2006).

Liver stem cells as precursors of HCC Oval cells, (stem cells which can differentiate into hepatocytes and bile ducts), may play a role as precursors to HCC, although the evidence is only circumstantial (Petersen, 2001). The question is whether bone marrow derived cells can act as precursors of oval cells. It has been proposed but refuted because bone marrow cells can fuse with liver cells and pose as oval cells (Thorgeirson, 2006). However, there remains evidence that extra hepatic cells can differentiate into liver tissue (Sun et al., 2009). Oval cells are not seen in normal liver and increase in number as cirrhosis progresses (Xiao et al., 2003). Many HCCs express markers of oval cells as do 50% of small cell dysplastic foci and hepatic adenomas. This supports the “cancer stem cell” theory (Libbrecht, 2008). Some HCCs contain cells that are ultra structurally identical to hepatic progenitor cells (Libbrecht, 2008). The idea is that cancers contain the same functional cell populations as normal tissues. Cancer stem cells retain the essential features of normal stem cells, i.e. the ability to self renew (Sell, 2009). They resist chemotherapy, persist and then the cancer stem cells reform the cancer (Sell, 2008). There is a parallel between HCC and the rodent oval cell response to the drug DDC, which induces tumor cell formation. Both HCC and the oval cell response involve Wnt/ β -catenin signaling (Apte et al., 2008).

Recently the phenotype of normal liver stem cells (liver progenitor cells) and liver cancer stem cells have been characterized. Both are positive for EpCAM, NANOG, Oct 3/Oct 4, SOX2, STAT3, ABCG2, WNT3a, β -catenin, CD90, CK18, CK8, CK7, ALB, CD44, and OV6. Both are negative for CD45. Less consistent correlations are BM11, Notch 1, Kit, CD133, CK19, AFP, CK14, and CD34. Interplay between WNT/ β -catenin, Hedgehog, Notch and TGF- β signaling pathways target and expedite tumorigenesis (Mishra et al., 2009). The Hedgehog signaling pathway normally regulates self-renewal for maintenance of the stem cell compartment and regulates cellular differentiation at least in the gut epithelium (Mishra et al., 2009) in association with Notch, sonic hedgehog and TGF- β signaling. The

TGF- β signaling pathway plays a key role in both HCC suppression as well as progression of differentiation from a stem or progenitor cell (Mishra et al., 2009).

Stem cell niches exist in normal human livers where specimens were obtained in resected livers for metastatic colon cancer. Patches of hepatocytes apparently stemming from portal tracks and projecting into the centrilobular venules were found to be deficient in cytochrome oxidase. Mutations were identified in the patches using mtDNA as a marker of clonal expansion. They were localized by immunohistochemistry (Mishra et al., 2009). The clonal expansions were not pathological, however.

We have noted scattered single stem cells in human cirrhotic livers, in hepatocellular carcinoma (HCC) and in prokaryotic stage 3 liver fibrosis. By *in situ* hybridization (FISH) the stem cells over express EGF, HGF and CK19. In immunofluorescent microscopy they over express OV-6 and GSTP. Since they appear in HCCs and cirrhotic livers they are candidates for preneoplasia and cancer cell renewal (Fig 1).

ANIMAL STUDIES ON MODELS OF PRENEOPLASTIC FOCI

Models of preneoplastic foci in animals go back many years (Thorgeirson et al., 2006) to the classical Solt and Farber sequential model of liver chemical carcinogenesis (Solt et al., 1977). Choline deficiency in combination with carcinogenic chemicals induces preneoplastic foci followed by liver neoplasms (Croager et al., 2002) as well as choline and folic acid deficiency (methyl deficiency diet) (Bagnyukova et al., 2008).

Markers used for identifying preneoplastic foci include glutathione S transferase placenta (GSTP) GSTmu, GSTpi (Inoue et al., 2008; Nishimura et al., 2008; Scolastici et al., 2008; Murae et al., 2008; Satoh et al., 2008; Fan et al., 2008; Perra et al., 2009; Sukata et al., 2004; Nagao et al., 1999; Roomi et al., 2006; Oliva et al., 2008; Oliva et al., 2009), diubiquitin (FAT10) (Nan et al., 2006; Evert et al., 2005) fatty acid synthase (FAS) (Tazawa et al., 1983; Nagao et al., 1998) gamma glutamyltransferase (GGT) (Perra et al., 2009; Oliva et al., 2009; Nan et al., 2006; Lee et al., 2003) $\alpha 2$ macroglobulin ($\alpha 2M$) (Nagao et al., 1999; Tazawa et al., 1983) and alpha fetoprotein (AFP) (Tazawa et al., 1983).

FAT10 positive cells initially appear singly, separated by normally stained hepatocytes and become reduced in number if the drug DDC or griseofulvin is withdrawn from the diet (Nan et al., 2006). However, if the drug is refed, the FAT10 positive cells rapidly increase in number over 6 days of feeding. They form MDBs and undergo mitoses (Nan et al., 2006). They show a growth advantage compared to the intervening normal hepatocytes. The number of PCNA and cyclin D1 positive nuclei increase in these cells (Nan et al., 2006; Oliva et al., 2009; Evert et al., 2005; Tazawa et al., 1983; Lukasiak et al., 2008). When the drug is permanently withdrawn after 10 weeks of feeding, the FAT10 positive cells are still found scattered in the hepatic lobules as single cells. 8 to 15 months after the drug is withdrawn some of the FAT10 positive cells occur in small to large clusters and form large tumors consisting of FAT10 positive cells where MDBs are sometimes formed (Oliva et al., 2009). (Fig 2).

FAT10 is also over expressed in human HCCs in 70% to 90% of cases (Teoh et al., 2008; Ren et al., 2006) and in livers and tumors in KU70 $-/-$ mice (Ren et al., 2006). One of FAT10's functions is to combine with MAD2 to prevent the delay of chromosomal spindle separation until all of the chromosomal kinetochores have connected to the microtubules during prometaphase. This causes chromosomal instability (Ren et al., 2006). However, no anaploidy was found in the tumors that formed in the DDC/FAT10 model (Oliva et al., 2008).

The FAT10 preneoplastic phenomenon resembles the resistant phenotype which selectively proliferates to form preneoplastic nodules in an altered adaptive response to toxic xenobiotics (Farber, 1990; Laconi, 2000). These FAT10 positive liver cells have been epigenetically altered to express a change in phenotype, which is heritable (Oliva et al., 2009). They have escaped the mitotic block imposed on normal hepatocytes by the drug. The FAT10 positive cells proliferate and replace the original parenchymal cells. The FAT10 positive cells/foci/tumors constitute the step wise process of cancer development. The FAT10 positive cells constitute initiated cells with a resistant phenotype toward cytotoxicity in the presence of a strong growth stimulus (Lukasiak et al., 2008).

The FAT10 induced initiation phenotype is the result of an epigenetic change in hepatocytes. This is based on the fact that the FAT10 phenotype change is prevented by the methyl donors S-adenosyl-methionine (SAME) and betaine (Li et al., 2008; Bardag-Gorce, 2008).

When DDC is refed in the DDC drug-primed model, the expression of 379 genes are changed (56). Feeding SAME with DDC prevents the up regulation of many of these genes present in all the functional pathways. The expression of FAT10 as well as KFL6, AFP and GSTm2 was prevented by SAME (Li et al., 2008). SAME prevented the changes induced by DDC refeeding by altering histone methylation of H3K4me3, H3K9me3 and H2A ubiquitination. It also activates the following enzymes involved in epigenetic modifications of gene expression i.e. GCNS, HDAC9, HDAC7a, SET 7/9 and SUV39H1 (Bardag-Gorce et al., 2008). Betaine feeding, like SAME, also prevented the DDC refeeding induced changes in gene expression. This included genes that affect methionine metabolism i.e. decreased MHTFr, increased MT1a, increased AHCY, GNMT, increased BHMT, increased AMD1, caused by DDC refeeding (Oliva et al., 2006). Betaine feeding prevented the decrease in S-adenosylhomocysteine caused by DDC refeeding (Oliva et al., 2006). SAME reduces or prevents the establishment of liver tumors but does not reduce tumors once they have become established (Lu et al., 2009; Pascale et al., 2002).

Microarrays were done on livers of rats treated with the 2 stage model of carcinogenesis to induce preneoplastic nodules. Rats were first treated with N-diethylnitrosamine initiation which was followed by feeding fenofibrate (FF) for 13 weeks. 2/3 hepatectomy was performed 1 week after FF was stopped. Microarray data was confirmed by RTPCR. Aco and Cyp4a1 (lipid metabolism), GPX2, Cat, Cyp2b15 and Ugt 1a6 (metabolic oxidative stress), Apex1, Mgmt, Xrec 5, Nbn and Gadd45a (DNA repair) and Cend 1 (cell cycle related) were up regulated by FF treatment. Cyp1a2, Gst2, Gstm2 and Gstm3 (phase 1 and metabolizing genes) Mih1 and Top 1 (DNA repair), and Cdkn1a, Cdkn1b, Chek2 and Gadd 45b (cell cycle/apoptosis) were down regulated by FF treatment (34). FF treatment increased carnitine acetyltransferase, acetyltransferase, palitoyltransferase, fatty acyl-CoA oxidizing system and catalase. The results support the conclusion that the changes developed by FF treatment induced preneoplastic foci resulting from oxidative stress (Ioue et al., 2008).

ANIMAL STUDIES ON PRENEOPLASTIC STEM CELLS

Based on the observation that alcohol synergistically enhances the progression of liver disease and the risk for liver cancer caused by hepatitis C virus (HCV), studies were conducted on the molecular mechanisms of this synergy (Machida et al., 2009). Alcohol fed HCV NS5A transgenic mice were fed ethanol by intragastric canula. The Lieber-DeCarli diet with ethanol was fed ad lib, in long term experiments. Toll-like receptor 4 (TLR4) was induced in the NSSA transfected mice. Alcohol feeding and this induction modified the synergetic liver damage and tumor formation, secondary to ethanol induced endotoxemia. Nanog, the stem/progenitor marker was up regulated downstream to TLR-4 signaling.

CD133/Nanog-positive cells were identified in the liver tumors that formed. Transplanted p-53 deficient hepatic progenitor cells transduced with TLR4 resulted in liver tumor formation in mice following repeated LPS injections. Concomitant transduction of Nanog short-hairpin RNA prevented tumor formation. It was concluded that Nanog mediates TLR-dependent liver oncogenesis by hepatic progenitor cells.

Rat strains susceptible to developing HCCs were compared with strains which were not susceptible to developing tumors (Feo et al., 2009). They were compared by mapping the genes responsible for inherited predisposition to form HCCs (Feo et al., 2009). These studies showed that the activity of several low penetrance genes and a predominant susceptibility gene regulate the development of hepatocarcinogenesis in rats. Analysis of the molecular changes showed an increase in iNos cross-talk with IKK/NFκB and RAS/ERK pathways in the most aggressive liver lesions of HCC in the susceptible F344 rats. ERK activity linked to proteasomal degradation of dual-specificity phosphatase 1, (Buspi), (a specific ERK inhibitor), by the CKS1-SKP2 ubiquitin ligase complex, was increased in the more aggressive HCCs of genetically susceptible rats.

Epigenetic phenomenon also played a role in HCC susceptibility: e.g. F344 HCCs exhibit global DNA hypomethylation, a change in the Mat1A/Mat2A ratio, reduced SAMe levels and changes in the methylation of CpG islands of Mat1A and Mat2A promoters. The Mat1A promoter was hypermethylated and the Mat2A promoter was hypomethylated in the susceptible F344 rats (Feo et al., 2009).

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Fig 1.
Confocal microscopy of cirrhotic livers with HCC was double stained with antibodies to OV6 (green) and GSTP (red). The merged photo shows co-localization of both proteins (yellow).

Fig 2. Livers from mice fed DDC for 10 weeks and withdrawn from the drug for 8 to 15 months still contained single FAT10 positive hepatocytes (arrow) and formed small aggregates of FAT10 positive hepatocytes as well as large tumors composed of FAT10 positive liver cells. Published with permission from the publisher Elsevier (ref 44).