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Invited Commentary

Potential roles of tumor suppressor genes and microsatellite instability in hepatocellular carcinogenesis in southern African blacks

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MAJOR POINTS OF THE COMMENTED ARTICLE

Cumulative loss of heterozygosity (LOH) of chromosomal regions and tumor suppressor genes has been reported in hepatocellular carcinomas (HCCs) from China, Japan, and Korea. In this issue of the World Journal of Gastroenterology, Martins *et al*[1] report an analysis of LOH and microsatellite instability in HCCs from a group of 20 southern African blacks. Six known tumor suppressor genes-p53, RB1, BRCA1, BRCA2, WT1, and E-cadherin-were analyzed for LOH. In addition, the p53 gene was analyzed for the codon 249 mutation that is commonly found in subjects exposed to high levels of dietary aflatoxin B1. The authors found LOH at the RB1 locus in 17% (3 of 18 informative subjects), at the BRCA2 locus in 10% (2 of 20 informative subjects) and at the WT1 locus in 8% (1 of 13 informative subjects). Two of the subjects had LOH at both the RB1 and BRCA2 genes. Thus, overall, LOH was found in 20%(4/20) of the HCCs. No LOH was found at the p53, BRCA1, or E-cadherin loci. In contrast to reports from other populations, mutations of the p53 and RB1 genes in combination were not seen in this population. Nine of 10 microsatellite loci examined showed changes in microsatellite repeat number in different HCCs, and changes at two or more loci were found in 15% (3/20) of the subjects. The p53 codon 249 mutation was found in 25% (5/20) of the subjects. Four of the 5 subjects with p53 codon 249 mutations had active or previous hepatitis B infection; the hepatitis B status of the fifth subject with a p53 codon 249 mutation was unknown. These results provide initial information about the potential role of specific tumor suppressor genes and low level microsatellite instability mechanisms in the pathogenesis of HCCs in southern African blacks.

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COMMENTARY

Different views of a single disease

HCC is a major cause of cancer death worldwide, particularly in Asia and Africa. The major risk factors for development of HCC are chronic hepatitis B virus and chronic hepatitis C virus infection, high dietary exposure to fungal aflatoxin B1, and other disorders causing cirrhosis such as hemochromatosis, alpha 1 antitrypsin deficiency, primary biliary cirrhosis, non-alcoholic steatohepatitis, and alcoholic cirrhosis. Over the past few decades, a number of approaches have be en explored in an attempt to elucidate the details of hepatocarcinogenesis. These approaches have increased in their sophistication with advances in cell and molecular biology and genetics and have borrowed from and contributed to our general understanding of tumor biology. Techniques and experimental systems that have improved our understanding of the hepatocarcinogenic process include: ① the use of chemical tumor initiators and promoters in animal models; ② studies of growth factors and their signaling pathways such as insulin like growth factor 2 (IGF-2) and its intracellular mediator, insulin receptor substrate 1 (IRS-1); ③ transgenic mouse models overexpressing cytokines, growth factors, or oncogenes such as tumor necrosis factor alpha (TNFalpha), transforming growth factor bet a (TGFbeta), or c-myc, expressed in isolation or in combination; ④ studies of immune-mediated mechanisms of hepatocellular injury; ⑤ analysis of the molecular genetic changes that occur in HCCs, including hepatitis B virus integration; and ⑥ studies of the protein products of the hepatitis B and C viruses and their interaction with host cell processes. More recently, there has been mounting evidence that common fragile sites, which are unstable regions in the genome, may also be involved in hepatocarcinogenesis.

These studies have contributed to our growing appreciation of the multiplicity of mechanisms and pathways that may contribute to the carcinogenic process in toxin affected, chronically inflamed or otherwise injured liver tissue. Unfortunately, we

are still far from a unified, comprehensive understanding of hepatocarcinogenesis. This is in part because HCC, although quite uniform in its final phenotype, is initiated in multiple genetic and environmental contexts and almost certainly emerges as a consequence of multiple possible pathways. However, the lack of a comprehensive view of the pathogenesis of HCC also prevents us from developing effective, targeted, preventive or therapeutic interventions that are elegant and also simple enough to be applicable to the vast majority of patients with this disease. In this commentary we will review current concepts of the mechanisms of human hepatocellular carcinogenesis and address the issue of geographic variation in carcinogenic mechanisms.

Tumor suppressor genes

Current concepts in molecular oncogenesis suggest that the chromosomal breaks and rearrangements or gene mutations found in cancer lead to activation of oncogenes or cause disruption of tumor suppressor genes. Tumor suppressor genes are normal cellular genes that, when homozygously deleted, can contribute to tumor development. Tumor suppressor genes generally operate in a recessive manner, requiring loss of both copies for tumorigenesis, in contrast to oncogenes, which can exert their effects in a dominant fashion. Loss of heterozygosity (LOH) is loss of one allele in a tumor cell from a chromosomal region for which the -individuals'-normal cells are heterozygous. LOH is detected using polymorphic DNA markers that can distinguish between the two alleles[2]. If one allele of a tumor suppressor gene is inactivated by mutation, then deletion of the second allele, detected by LOH, is associated with loss of expression of the tumor suppressor gene. In human HCCs, LOH has now been reported in multiple chromosomal regions, including 1p, 1q, 2q, 4q, 5q, 6q, 8p, 8q, 9q, 10q, 11p, 12p, 13q, 14q, 16q, and 17p[3,4]. In a number of these regions, there are known or putative tumor suppressor genes, such as at 17p13.1 (p53), 6q26-27 (mannose-6-phosphate/ insu lin-like growth factor 2 receptor), 8p21.3-22 (DLC-1), and 13q12-q32 (RB and BRCA2). For a few of these chromosomal loci, clinico-pathologic associations have been demonstrated, such as the association of LOH on chromosome1p with early stage HCC[5]and that of LOH on chromosome 16q with progression of HCC[6]. In addition, patients with LOH at multiple regions have more advanced stage disease, less well-differentiated tumors, higher serum alpha-fetoprotein levels and a worse prognosis[7]. Chronic hepatitis B viral infection and in tegration of the hepatitis B genome have also been associated with high rates of genomic instability[8,9]. The use of the technique of comparative genomic hybridization (CGH) allows the identification of gains and losses of DNA sequences across the entire tumor genome. Analysis of HCC tumors and cell lines by CGH has revealed genomic DNA copy number gains at 1p34. 3-35, 1p33-34.1, 1q21-23, 1q31-32, 6p11-12, 7p21, 7q11.2, 8q24.1-24.2, 11q11-13, 12q11-13, 12q23, 17q11. 2-21, 17q23-24, and 20p11.1-q13.2. Recurrent losses were found at 3p12 -14, 3q25, 4p12-14, 4q13-34, 5q21, 6q25-26, 8p11.2-23, 9p12-24, 11q23-24, 13q12-33, 14q12-13, 15q25-26, 16q, 17p, 18q11.2-22.2, and 21q21-22[10,11]. Significantly, a majority of the over-represented regions harbor know n proto-oncogenes, and half of the under- represented regions coincide with sites of known or putative tumor suppressors[11]. Notably, the recognition that there is gain of region 17q11-21, which harbors the ERBB-2 proto-oncogene, has led to the development of early clinical trials of anti-p185HER2/neu monoclonal antibody in appropriately selected HCC patients.

Investigation of p53 gene mutations in HCC has been particularly revealing, as HCCs from patients with high dietary exposure to fungal aflatoxin B1 have a high frequency of point mutations at the third position of codon 249 of the p53 gene, resulting in a G:C to T:A transversion[12]. This mutational target appears to be specific for liver tumors of hepatocellular origin. A G to T transversion at the second position of codon 249 is commonly found in patients with chronic hepatitis B and C infection. The occurrence of this mutation correlates with oxyradical exposure. Both of these mutations lead to decreased binding of p53 to its nuclear DNA targets[13]. p53 mutations have been demonstrated in nonneoplastic liver cells in subjects from communities with high dietary AFB1 exposure, suggesting that the mutations are early events in neoplastic transformation. However, p53 mutations can also occur late in tumor progression. The hepatitis B virus HBx gene product has also been shown to interact with p53 and strongly inhibit p53 sequence-specific binding, leading to inhibition of p53-mediated apoptosis. Abnormalities in the retinoblastoma gene have been noted in association with p53 mutations in advanced HCCs, particularly in poorly differentiated tumors, and a possible additive effect of p53 and Rb mutations during the progression of hepatocarcinogenesis has been suggested[14].

Oncogenes

Proto-oncogenes are cellular genes involved in the control of cell growth. Mutation, overexpression, or amplification of these genes leads to oncogenic activity that contributes to neoplastic transformation. The oncogenes c-fos and c-myc are both overexpressed in HCC[15]. c-myc overexpression may be a conse quence of amplification of the c-myc locus in HCC[10,11]. Activation of the c-myc and c-fos also occurs as a consequence of the transactivating function of the

hepatitis B virus x protein (HBx) and the carboxyterminal truncated middle hepatitis B surface protein (MHBs-t)[16]. Oncogene activation may occur through reactive oxygen species mediated pathways or through protein kinase C or mitogen activated protein kinase pathways. Reactive oxygen species or lipid peroxidation mediated processes may also be important in the pathogenesis of chronic liver injury induced by alcohol, genetic hemochromatosis, and alpha 1 antitrypsin deficiency[17,18].

Growth factors and growth factor signaling pathways

Insulin and insulin-like growth factors (IGF) 1 and 2 promote hepatocyte growth . IGF-2 is frequently over expressed in HCCs. In addition, insulin receptor sub strate 1, a cellular mediator of insulin-like growth factor signaling, is also frequently overexpressed in HCCs[19]. Signaling events downstream of IRS 1 lead to cell proliferation through upregulation of cellular growth genes and inhibition of apoptosis. Transforming growth factor alpha (TGF alpha) and the structurally related epidermal growth factor (EGF) are another potent class of hepatocellular growth factors. TGF alpha is overexpressed in the liver of patients with chronic hepatitis. As TGF alpha levels are often increased in HCCs, it is likely that it contributes to cellular proliferation in cancer, and may be a factor in tumor initiation or progression in patients with chronic hepatitis[20]. Transforming growth factor beta 1 (TGF beta 1) inhibits cell proliferation and promotes cellular differentiation, fibrogenesis, and apoptosis. Increased TGF beta 1 levels may create an environment in which selection of hepatocyte clones resistant to TGF beta 1-induced apoptosis occurs.

Microsatellite instability

Microsatellite instability (MSI) is defined as a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumor when compared to normal tissue. This form of genomic instability is associated with defective DNA mismatch repair in tumors, which is important in the pathogenesis of the hereditary non-polyposis colon cancer syndrome (HNPCC) and associated malignancies[21]. To date, six mismatch repair genes have been identified in humans; hMLH1, hMSH2, hPMS1, hPMS2, hMSH3 and hMSH6. Colorectal cancers may be characterized as having high-frequency MSI (MSI-H) if greater than 30%-40% of more than 5 microsatellite loci analyzed show instability, low-frequency MSI (MSI-L) if less than 30%-40% of more than 5 loci analyzed show instability, or as being microsatellite stable (MSS) if 0% of loci show instability[21]. Of sporadic colorectal cancers, 70% are MSS, 15% are MSI-L, and 15% are MSI-H. MSS or MSI-L colorectal cancers do not have an associated defective mismatch re pair phenotype or the clinico-pathologic features of HNPCC tumors; instead they behave in the same manner as sporadic colon cancers. Clear criteria have not be en defined for MSI in noncolonic tumors. To date, a number of studies, including the study by Martins *et al*[1], have demonstrated a relatively low f requency of low-level MSI in HCC[22,23]. As is the case with other tumors, it is unclear whether this finding is of significance in the etiology or pat hogenesis of HCCs or is instead simply a consequence of the generalized genomic instability found in cancer. It has been suggested that cumulative low-level MSI at multiple loci leads to tumor progression. Further investigation is needed to res olve this potentially important question.

Chromosomal fragile sites

Chromosomal fragile sites are specific genetic loci which are susceptible to forming gaps, breaks, and rearrangements in metaphase chromosomes of cells cultured under conditions that inhibit DNA replication, such as treatment with the DNA α polymerase inhibitor, aphidicolin. Fragile sites are grouped into “common” or “rare” classes based on their frequency of occurrence and the culture conditions required for their expression. Thus far, 89 common and 28 rare chromosomal fragile sites have been identified. Common fragile sites are present in all individuals. The most frequently observed common chromosomal fragile sites occur at 3p14.2 (FRA3B), 16q23 (FRA16D), 6q26 (FRA6E), 7q32 (FRA7H), and Xp22 (FRAXB)[24].

Common fragile sites span a distance of 250-500 kilobases and display characteristics of unstable, highly recombinogenic DNA *in vitro*. In particular, they are preferred sites for sister chromatid exchanges, chromosomal deletions and rearrangements, integration of viral sequences and transfected plasmid DNA, and in itiation of bridge-breakage-fusion cycles, which lead to gene amplification. Due to these characteristics and the frequent coincidence of fragile sites with chromosomal breakpoints in malignant cells, it has been hypothesized that fragile sites are involved in carcinogenesis[25]. The most convincing evidence of the potential significance of the fragile sites in carcinogenesis is the location of FRA3B, the most highly inducible common fragile site, at chromosome 3q14.2. This chromosomal region is frequently deleted in lung cancer, renal cell carcinoma, and pancreatic cancer, and is also the location of the fragile histidine triad (FHIT) gene[26]. The FHIT gene has been proposed to be a tumor suppressor and has recently been identified as a preferential target in HCC[27]. The cloning of additional fragile sites, particularly ones such as FRA16D (16q23) and FRA6E (6q26) that are located in regions at which there is known LOH in HCC, should provide additional information about

the potential role of fragile sites in hepatocarcinogenesis.

Telomere length and telomerase activity

Telomeres are specialized protein-DNA structures at the ends of chromosomes that contain long stretches of TTAGGG hexameric repeats. Telomeres are thought to prevent degradation of chromosome ends and end-to-end fusion with other chromosomes. Aging of somatic cells is associated with reduction in telomere length. In contrast, germ line and neoplastic cells express telomerase, an enzyme that restores telomere length. There is progressive shortening of telomeres with progression from chronic hepatitis to cirrhosis and eventually to HCC[28]. This is thought to occur as a consequence of the multiple cycles of cell injury, death, and regeneration that occur in injured liver, leading to premature hepatocellular senescence. It is presumed that telomere shortening beyond a critical length leads to genomic instability of hepatocytes and the evolution of clones of hepatocytes with increased telomerase expression and an immortalized phenotype[29].

Geographic variations in hepatocarcinogenic mechanisms

Because of the small number of subjects in many studies and the heterogeneity of etiologies of HCC, it has been difficult to conclusively identify geographic variations in hepatocarcinogenic mechanisms. The best evidence so far is for the role of aflatoxin B1 in generation of the G-to-T transversion at codon 249 of the p53 gene. The frequency of this mutation in HCCs increases proportionally to the level of dietary exposure to aflatoxins. LOH at different loci, includin g the p53 locus, does not show as clear a pattern. Fifty-eight % of 64 heterozy gous Chinese patients had a tumor-specific p53 allele LOH[30]. Reported rates of p53 LOH from Japan are 69% (55 of 80 informative cases) and 95% (34 of 36) in cases with a p53 mutation[31]. Previously high reported rates of p53 LOH in HCCs from southern African blacks contrast with the results reported in this issue by Martins *et al*[1,32]. It is unclear whether this represents an artifact due to the small numbers in both studies or is reflective of a real phenomenon. Higher rates of LOH of the retinoblastoma gene than were found in this study have also been previously found in HCCs from Korea, Japan, and Australia. In addition, coincident mutation of the p53 and RB1 genes has been observed in 25% of advanced HCCs from Japan and 12.9% of advanced HCCs from Australia.No coincident mutations of the p53 and RB1 genes were identified in this population of southern African blacks. For the BRCA1 gene, LOH has been reported only once, in 11.5% (3 of 16) HCCS in a Korean population. LOH of the region containing the E-cadherin gene has been reported in 64%-91% of HCCs from China and Japan[6,33]. No LOH in this region was found in the study by Martins *et al*[1]. Further study of larger numbers of advanced HCCs from southern Africa will be needed to confirm the possibility of a significant geographic variation. Homozygous deletions of the tumor suppressor gene p16/CDKN2A, which is located on chromosome 9p, have been shown to be frequent in HCCs from South Korean patients (61%) but uncommon in HCCs from Australian and Japanese patients. CGH analysis of a small number of HCC cell lines derived from different geographic regions showed loss of the 9p12-14 region in all 6 of 6 HCC cell lines established in South Korea[11]. This may reflect an association between hepatitis B virus infection, the major risk factor for HCC in Korea, and deletions of 9p.

In general, studies of different groups of patients from the same country have shown fairly high rates of variability of LOH. It appears that some patient populations have (or experimental methods result in) lower levels of LOH overall than other patient populations (or methods). In spite of this variability, there is remarkable consistency in the chromosomal locations of LOH across tumors and populations, suggesting that these are targets of the carcinogenic process of represent areas that are preferentially affected by genomic instability in HCC.

SUMMARY

Studies such as the one reported here by Martins *et al*[1], in which limited numbers of HCCs are examined at a limited number of loci provide tantalizing clues to the potential genetic mechanisms of hepatocellular carcinogenesis, and point out areas where further work is needed. A few groups have recently reported studies with comprehensive coverage of the genome with several markers on each chromosome. However, even the most detailed of these genomic screens has used approximately twenty markers per chromosome. For chromosomes that have 100 megabases of DNA sequence, this translates into a marker every 5 megabases. Since the average gene stretches over from less than ten to a few tens of kilobases, it is clearly impossible with current technology to achieve a comprehensive mapping of all gene deletions or rearrangements occurring in HCC. However, the advent of techniques such as comparative genomic hybridization and array based genomic DNA and RNA expression technologies has greatly expanded our ability to determine the genomic and expression differences between normal, precancerous, and can cerous tissues. Studies comparing genomic and gene expression changes in enough patients from different geographic regions on whom detailed clinical information is available will be invaluable in improving our understanding of the role of particular genomic targets and changes in gene expression in the development of HCC. A number

of secular trends will also affect our ability to elucidate the path ways underlying development of HCC. First, there is a trend towards using ablative therapies and liver transplantation for treatment of HCC and away from the use of surgical resection. This trend, if it continues, may reduce the availability of tissue samples for use in research. It is therefore important that priority be given to the establishment of tissue banks and databases of clinicopathologi c information on patients with HCC. Second, it is clear that few research groups have the clinical capacity and resources to independently perform the basic, translational, and clinical research initiatives that are needed to address the pressing questions of prevention, diagnosis, and treatment of HCC. There is therefore a clear need for the formation of international collaborative groups to cooperate in determining the molecular pathogenesis of HCC, to help us better understand the geographic differences in the pathogenesis of HCC, and also to collaborate in developing effective, technologically appropriate preventive, diagnostic and therapeutic alternatives. Major steps in this direction have included the development of joint projects between scientists in China, Africa and the United States and Europe and further collaborations should be actively encouraged.

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