Molecular Signatures of Environmental Mutagens in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the 5th most common cancers, with 80% of the cases occurring in low-resource countries. Its etiology is dominated by complex interplay between chronic infection by hepatitis virus B or C (HBV, HCV), metabolic diseases and exposure to environmental carcinogens. In areas of high incidence of HCC, the most common risk factors are chronic HB carriage and exposure to a mycotoxin, aflatoxin B1 (AFB1), which contaminates many staples and causes mutations at the third base of codon 249 in the TP53 tumor suppressor gene in hepatocytes (R249S mutation). In this review, we summarize studies using a very sensitive and quantitative detection method, short-oligonucleotide mass analysis, to measure R249S in cell-free DNA isolated from the plasma of asymptomatic subjects and patients with chronic liver disease and/or HCC. These studies have identified that high levels of R249S were strongly associated with HBV-related HCC. Low to intermediate levels of R249S, in contrast, were detectable in asymptomatic subjects exposed to AFB1, with seasonal variations informative of the complex interactions between mutagenesis by AFB1 and chronic infection by HBV. Overall, we suggest that formation of R249S occurs in response to AFB1 exposure, well ahead of cancer development, thus generating large populations of cells at high risk for neoplastic transformation. In addition, R249S mutations may inactivate pro-apoptotic activities of p53 and contribute to rendering hepatocytes resistant to liver cell destruction by chronic inflammation, thus limiting chronic liver disease symptoms.

Key words: mutations, TP53, plasma DNA, aflatoxin, short-oligonucleotide mass analysis, liver cancer

Introduction

Liver cancer is a paradigm for environmental carcinogenesis. Most liver cancers in adults fall into two major categories; hepatocellular carcinoma (HCC) and cholangiocarcinoma (CAC), whereas in young children, the main form of liver neoplastic disease is hepatoblastoma. HCC represents about 80% of all cases of liver cancer and shows high incidences in many parts of Sub-Saharan Africa, Eastern Asia and in some regions of Central and Latin America. This cancer results from the transformation of hepatocytes, the cells that support the metabolic functions of the liver. CAC develops from intrahepatic biliary epithelial cells and is relatively rare except in areas where chronic infections of biliary ducts by liver flukes (Opisthorchis viverrini, Clonorchis Sinensis) are endemic (Thailand, Laos, Vietnam, South-Eastern China) (1). Both cell types originate from a common precursor in the embryonic liver, which can differentiate either in hepatocytes or in cholangiocytes (2).

Overall, the annual number of new liver cancer cases is estimated at 7–800,000, representing the 5th most common form of cancer. It is however the 3rd cause of death by cancer worldwide due to its very low cure rate, in particular in low-resource countries where this cancer is particularly frequent (3). The vast majority of liver cancers are associated with environmental causes. Heredity only plays a minor role, except in the case of relatively rare metabolic diseases such as haemachromatosis and tyrosinaemia type II (4). Most liver cancer cases are sporadic and develop within the framework of interplay between infectious agents (viruses or parasites), metabolic conditions (enhanced by alcohol addiction or metabolic liver syndromes) and exposure to mutagens (including in particular dietary carcinogens). The main etiological agents of HCC are hepatitis viruses B (HBV) and C (HCV) which are responsible for, respectively, about 50% and 25% of HCC cases worldwide (5). Other factors include persistent liver injury caused by alcohol addiction or by metabolic syndromes such as non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH) (6). The most significant dietary carcinogen is aflatoxin B1 (AFB1), a mycotoxin produced by fungi that infects many dietary staples in hot and humid climates (7). Additional factors are iron overload due to contamination...
radiation with $^{232}$Th contained in Thorotrast, a contrast agent used in radiological diagnosis until the mid–20th century (6).

In this review, we briefly summarize current knowledge on the roles of environmental mutagens in HCC and we describe recent studies on the detection of AFB1-induced mutations in DNA isolated from the plasma or serum.

Pathogenesis of Hepatocellular Carcinoma

In many instances, HCC develops in the context of pre-existing liver cirrhosis, a chronic liver disease characterized by chronic inflammation, destruction of hepatocytes, compensatory cell proliferation and progressive formation of liver fibrosis. HCC develops in a stepwise process, starting from chronic deregulation of liver functions as the result of persistent liver damage resulting from viral infections, metabolic perturbations, chemical damage and other forms of hepatocyte stress.

Chronic liver damage induces the rapid destruction of many mature liver cells through apoptosis or necrosis. The cause of liver damage is inflammation resulting in the production of reactive oxygen and nitrogen species that have devastating consequences on growth and survival signaling pathways, accumulation of mutations through generation of free radicals, and activation of stellate cells. Moreover, viral replication causes endoplasmic reticulum (ER) stress resulting in further oxidative stress. Together, these processes stimulate liver regeneration through a compensatory cell proliferation process. This proliferative activity provides a basis for cellular changes leading to cirrhosis (Fig. 1).

Precancerous lesions of HCC include cytological changes indicative of dysplasia, microscopically detectable as expansile foci and nodular lesions, detectable on gross examination or by imaging, characterized by cytological or structural atypia (dysplastic nodules, DN). Depending upon the degree of atypia, DN is further classified as low-grade (LGDN) or high-grade (HGDN). One of the features that distinguish HGDN from LGDN is the degree of neovascularization (“unpaired arteries”). Early HCC sometimes occur as a sub-nodule with HGDN. Early HCC is a poorly defined nodular lesion measuring less than 2 cm, with well-differentiated microscopic features, increased cell density and irregular pattern of growth. Of note, early HCC is sometimes difficult to distinguish from HGDN, in particular on biopsies. On the other hand, small size (less than 2 cm) is not by itself a criterion for early HCC. Distinctively nodular HCC, in particular, presents as small nodules with cellular features similar to those of larger HCC. On the other hand, cellular characteristics of early HCC may sometimes be observed in tumors >2 cm, suggesting that some HCC may have a slow cellular and biological evolution. In Western countries, it is estimated that about 90% of HCC are preceded by a diagnosis of liver cirrhosis (8). However, in low resource countries where HCC is frequent, it is often diagnosed at an advanced stage with no evidence of pre-existing liver cirrhosis. In a recent study in The Gambia, West Africa, it was found that only about 65% of the cases had detectable cirrhosis in the liver at the time of diagnosis, most of which might result from damage to the normal liver generated during the progression of cancer (9). Estimates from the national registry of chronic liver disease indicate that less than 15% of HCC cases have a diagnosis of cirrhosis that precedes HCC (E. Bah, personal communication, 2008). Moreover, at the time of HCC diagnosis, patients report an average duration of liver symptoms of less than 3 months. In Thailand, a cohort study conducted in 1988–1995 by Dr. Petcharin Srivatanakul at the National Cancer Institute, Bangkok in collaboration with the International Agency for Research on Cancer (IARC) has observed that only 50% of chronic HBV carriers who developed HCC during cohort follow-up had a diagnosis of cirrhosis before detection of HCC (unpublished results). Thus, in regions of high incidence of HCC, liver cirrhosis does not appear...
to be a prerequisite to the development of liver cancer, in contrast with lower incidence areas.

**Molecular Mechanisms**

HCC develops through multiple genetic pathways depending upon the combination of risk factors involved. The two most common genetic alterations occur in TP53 (encoding the tumor suppressor protein p53; 30–70%) and in components of the oncogenic Wnt/β (beta)-catenin pathway (about 50%). Other affected genes include regulators of the TGFβ (beta) signalling pathways (SMAD2, SMAD4), IGF2 receptor (IGF2R) and genes involved in growth control through the RB1 (retinoblastoma) pathway. Laurent-Puig and Zucman-Rossi have proposed that HCC may fall into two broad molecular categories (10). The first, characterized by chromosome instability, contains HCC occurring in a context of chronic infection by HBV with TP53 mutations; these tumors often show a poorly differentiated phenotype. The second, characterized by chromosome stability, is more common among non-HBV HCC, and their hallmarks include mutations in the Wnt/β (beta)-catenin pathway.

HBV is a partially double stranded DNA virus which contributes to HCC by at least three specific mechanisms. First, the HBV genome can integrate into the genome of chronically infected hepatocytes. Integration of HBV, often in a partial form, is detectable in all cases of HCC associated with HBV. HBV genome integration has been associated with host DNA micro-deletions in genes such as telomerase reverse transcriptase (TERT), platelet-derived-growth-factor receptor-β (PDGFRβ) or mitogen activated protein kinase 1 (MAPK1), among others (11–14). Second, the viral oncoprotein HBx interacts with many cell factors and modulate their activity, including components of SRC, RAS, RAF, MAPK, ERK, JNK and NF-kappaB signaling cascades (15–17). Third, HBx binds and perhaps inactivates the tumor suppressor protein p53, therefore increasing cellular proliferation and survival and compromising DNA-damage checkpoints (16,18,19). The effect of HBV on the pathogenesis of liver diseases and HCC differs according to the genotype of the virus and to the presence of mutations in specific regions of the viral genome (reviewed in (20)). There are currently 8 distinct HBV genotypes (defined as virus strains that differ by at least 8% of their genomic sequence). The B and C genotypes common in Asia appear to be associated with a greater risk of progression to chronic liver disease and cancer that the D genotype encountered in most chronic infected subjects in the Middle East or in India (21).

HCV is an RNA virus without a DNA intermediate form which, in contrast to HBV, does not integrate into the host genome (22,23). HCV is thought to act mainly by an indirect mechanism involving cycles of hepatocyte destruction by inflammation associated with immune response to the virus and subsequent regeneration, which favors the development of liver cirrhosis and facilitates the accumulation of mutations. On the other hand, the viral protein NS5A has been proposed to inactivate p53 by blocking it in the peri-nuclear space (24). Many patients may be co-infected by HBV and HCV, often with HBV in an occult form (serologically silent but detectable at the DNA level) (25,26).

Aflatoxin B1 (AFB1) is produced by the fungi _Aspergillus flavus_ and _Aspergillus parasiticus_ that contaminate staples in regions such as Sub-Saharan Africa and South-East Asia, including groundnuts (peanuts), maize and corn. AFB1 is metabolized in the liver into an epoxide that covalently binds to the N7 position of guanine at the third base of codon 249 in the TP53 gene (AGG, encoding arginine), resulting in a transversion mutation which is extremely rare in cancers other than liver (AGT, encoding serine, R249S mutation, mutant protein p.R249S) (27,28). There is a strong ecological concordance between high exposure to AFB1 and a high proportion of R249S-positive HCC (up to 70% in parts of China and Africa). AFB1 acts synergistically with HBV in causing HCC. Subjects with chronic HBV and exposure to AFB1 have a 3–15-fold greater risk of developing HCC than subjects exposed to either factor alone (29). However, the molecular basis for this synergy is still poorly understood. Gouas et al. have shown that p.R249S forms a complex with HBx and that this complex seems to enhance proliferation in a HCC cell line (19). In another study, Jiang et al. have shown that tumor-derived HBx mutants in cooperation with p.R249S could increase cell proliferation and chromosome instability in normal human hepatocytes (18).

Chronic alcohol consumption generates massive metabolic stress in hepatocytes and enhances the development of metabolic diseases (6). Alcohol induces the activation of Kupffer cells, which release chemokines and cytokines with deleterious effects on hepatocytes. Moreover, several metabolites of alcohol such as acetaldehyde may damage DNA and induce mutations, although "signature" mutations of acetaldehyde have yet to be identified in HCC.

Iron overload in the liver occurs in subjects with genetic defects in iron absorption (hemachromatosis) and in subjects who consume iron-contaminated food, e.g., due to the use of iron utensils for cooking (30). Iron overload causes extreme oxidative stress in liver cells due to the iron-catalyzed Fenton reaction. The resulting reactive oxygen species induce DNA damage (strand breaks, base oxidation) and generate an inflammatory context resulting in chronic hepatocyte destruction/regeneration, stellate cell activation, development of cirrhosis and ultimately HCC (31). Recent evidence in mouse models suggests that iron overload may up-
regulate p53 expression by down-regulation of mRNA levels of MDM2. This may therefore increase the suppressor activity of p53, thus leading to enhanced apoptotic cell death. Escape from p53-dependent cell death, e.g., through inactivation of p53 by mutation, may bypass suppression and leads cells towards uncontrolled proliferation (32).

Searching for Mutagen Signatures: The Case for Plasma DNA

The brief overview above illustrates the critical roles of mutagenic processes in the pathogenesis of HCC. These roles can be categorized into two main families of processes. First, mutagenesis may result from the overproduction of reactive oxygen or nitrosative stress may modify DNA methylation patterns at sites targeted by carcinogens, thus modulating the formation of DNA adducts (33). Identifying mutations at early stages of progression from chronic liver disease to cancer may thus provide very interesting biomarkers for detecting ongoing mutagenic processes. Current methods for mutation detection are suitable for identifying specific mutations in minimal biological samples (e.g., fine-needle aspirates or small biopsies) (34). However, obtaining such materials is not straightforward in clinical practice and is often extremely impractical in low-resource contexts where many HCC cases occur. To address this difficulty, we and others have pioneered the use of free circulating DNA (CFDNA) isolated from the plasma as a surrogate source of liver-derived DNA (for a review of technical aspects of CFDNA see (35)).

The plasma or serum of healthy subjects contains minute amounts of CFDNA released in the bloodstream by many organs and tissues. It is estimated that, on average, normal blood may contain about 1 to 10 nanograms of DNA per mL of plasma. Levels of CFDNA are markedly increased in subjects with chronic inflammatory diseases and, significantly, in subjects with cancer (Table 1). Pathologies of the liver appear to be particularly prone to the release of CF DNA in the bloodstream. The liver is a massive organ (0.6–1.3 dm³), made up of 80% of parenchymal cells, which contains an estimated number of 1–3 × 10¹¹ hepatocytes. Normal, quiescent hepatocytes have a slow turnover rate (half-life: 6 months) but undergo rapid and vigorous proliferation in response to liver cell death. Thus, the daily turnover of hepatocytes can be conservatively estimated at about 1–5 billion cells per day, representing a total amount of genomic DNA of about 6 to 30 mg per day. Most of this DNA is supposed to be degraded locally through apoptosis or resorption of senescent cells. However, since the bloodstream is the major, if not the sole outlet for biological products shed by the liver, it is conceivable that part of this DNA may end up in the circulation. The amount of CFDNA detected in healthy subjects is equivalent to about 0.1–0.2% of the estimated cell DNA turnover in the liver. Noteworthy, many organs and tissues with rapid turnover such as the digestive and respiratory tracts release degraded materials by shedding into external spaces. Thus, although CFDNA may originate from a wide range of tissues and cell types, it is likely that liver is a major contributor to the overall amount of CFDNA.

The nature of CFDNA is still a matter of controversy. Naked DNA is unstable in blood and it has been proposed that CFDNA might be somehow protected from degradation by plasma DNases (36,37). We have used gel electrophoresis to determine the size of CFDNA in the plasma of normal subjects in comparison with patients with liver cirrhosis or liver cancer. This analysis revealed that in subjects with cirrhosis, CFDNA was essentially made up of apoptotic DNA fragments forming a typical DNA ladder. In contrast, in normal subjects and in HCC patients, most of CFDNA was detectable as a high molecular weight material compatible with genomic DNA (35). Thus, in patients with cirrhosis, most of the CFDNA may occur as a consequence of massive hepatocyte apoptosis. In contrast, in healthy subjects and in HCC patients, the presence of DNA with an apparent genomic size suggests that it is made of entire genomes shed in the bloodstream. It is therefore likely that a significant proportion of CFDNA is bound to proteins, possibly as nucleosomes, providing CFDNA with some form of protection against degradation in the plasma.

The mechanism of CFDNA clearance from plasma is poorly understood. By measuring the turnover of the Y chromosome in the plasma of mothers (as marker for placental recirculation of foetal cells or DNA in the mother’s blood) in the hours following the delivery

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Data compiled from Gormally et al., 2007 (35).
*Controls from European Prospective Investigation on Cancer (EPIC), a prospective study on healthy subjects recruited in 23 centers in Europe (see Gormally et al., 2006 (52)).
of a boy, Lau et al. have estimated the half-life of CFDNA to about 30 minutes (35). Increased amounts of circulating DNA in the blood of patients may reflect a disturbance of the equilibrium between CFDNA release and clearance. In any case, current evidence suggests that high levels of CFDNA are maintained through active and continuous release rather than through progressive accumulation over a long period of time.

**Mutation Signature for Aflatoxin Exposure in HCC**

Studies by Groopman and collaborators and by us have investigated the use of CFDNA as a source of material to detect R249S mutations associated with aflatoxin exposure in the plasma or serum of subjects from regions of high incidence for HCC (reviewed in (28,38,39))

In 2000, we have conducted a case-control study in The Gambia, West Africa, to assess the association between detection of R249S and risk of HCC. In this country, chronic HBV infection is endemic, affecting 11% to 16% of the adult population. AFB1 exposure is also widespread, and the annual incidence of HCC peaks at 80 cases per 100,000 among 35–55 year-old males (40,41). We found that R249S was detectable in the plasma of 40% of HCC patients, with a concordance of 88% between plasma and tumor DNA (40,42,43). R249S was also found in 15% of patients with liver cirrhosis and in 3% of control subjects with no detected liver disease (42), indicating that detection of the mutation was not restricted to patients with HCC or with its precursor disease, cirrhosis. Detection of R249S in CFDNA in a small proportion of controls raises the question of whether this mutation may represent a biomarker of ongoing exposure to aflatoxin and whether its quantitative assessment may elucidate the question on the progression towards HCC.

To address these questions, we have adapted and used electro spray mass spectrometry (short oligonucleotide mass analysis, SOMA), a method initially developed in the laboratory of J Groopman (Johns Hopkins University, Baltimore, USA) (44). This method is based on PCR amplification of exon 7 of TP53 in CFDNA using primers that contain restriction sites allowing cleavage of the PCR product into short oligonucleotides (8-mers) that encompass codon 249. These oligonucleotides are then purified by high-performance liquid chromatography (HPLC), electro spray ?ionized and analyzed by tandem MS/MS to generate small fragments with masses characterizing the G to T transversion at the third base of codon 249 (45). By using a plasmid containing a modified sequence of TP53 exon 7, we have generated an internal standard for expressing R249S levels in numbers of copies of DNA per mL of plasma. Using samples from the case-control study in The Gambia, plasma concentrations of R249S were increased in HCC cases (median: 2,800 copies/mL, range 500–11,000) compared to controls (median: 500 copies/mL, range 250–2,000). However, low levels of R249S (>500 copies/mL) were detected in 46% of asymptomatic controls, which suggests that the mutation may also serve as a marker of ongoing exposure to aflatoxin (45).

To determine the relationship between R249S in CFDNA and consumption of aflatoxin- contaminated food, we used SOMA to analyze R249S levels in CFDNA in a cross-sectional survey of 473 asymptomatic subjects (237 HBV carriers and 236 non-carriers) recruited in three rural villages in The Gambia over a 10 months period. These villages have been previously surveyed for food consumption habits and studies on individual levels of AFB1-albumin adducts in serum or urine have shown a seasonal pattern of exposure, consistent with high exposures in the months that immediately follow the harvest of groundnuts (peanuts), the main source of AFB1 exposure (December to March) (46,47). Comparison between subjects recruited at different times of the year detected a seasonal variation in R249S levels. Positivity for R249S (61%) and average concentration (5,690±11,300 R249S copies/mL serum) were significantly higher in HBV surface antigen (HBsAg)-positive subjects surveyed during April-July than in October-March (32% and 480±1,030, respectively) (proportion: OR: 3.59, [95% CI (2.05–6.30)]; concentration: p<0.001) (Fig. 2) (48). HBeAg positivity (a marker of HBV replication) and viral DNA load also varied seasonally with 15–30% of subjects surveyed between April and June being HBe-positive, compared with <10% surveyed during other months. These
results demonstrate that levels of \textit{R249S} in CFDNA of asymptomatic subjects are strongly influenced by HBV chronic infection status. Moreover, levels of \textit{R249S} show a seasonal variation that does not match the variations in exposure to AFB1: the peak of \textit{R249S} in the plasma occurs 3–6 months later than the previously reported peak of exposure to AFB1 (assessed by measuring AFB1-DNA adducts). This difference may be the consequence of a chain of molecular events determined by the turnover of hepatocytes. Mature hepatocytes metabolize AFB1 and may acquire DNA adducts during the period of high exposure to the toxin (Decem-
ber–March).

Subsequently, mutations may occur when cells are induced to re-enter proliferation in response to liver damage. Release of \textit{R249S} may occur when cells having acquired a mutation are eliminated as a result of normal turnover or of a seasonal flare of cell destruction. In this respect, it is important to note that increase in \textit{R249S} levels are detected in April–June, 4 to 6 months later that the reported increase in DNA adducts. This time difference is shorter than the estimated half-life of normal hepatocytes (6 months), suggesting that additional factors may cause hepatocyte destruction and release of \textit{R249S} in CFDNA. The observation of a seasonal peak of HBV replication just before the increase in serum levels of \textit{R249S} raises the hypothesis that the shedding of \textit{R249S} in CFDNA may be caused by a flare of HBV that induces an increase in cell turnover.

Changes in levels of \textit{R249S} in CFDNA are also detectable during the progression of chronic liver disease. In a prospective study in China, \textit{R249S} was detected in the plasma of asymptomatic HBV carriers several years before HCC diagnosis (49). In a subsequent study in a cohort of chronic carriers in the region of Qidong, China, we showed that levels of \textit{R249S} in the plasma were inversely correlated with the time to HCC diagnosis, suggesting that a steady increase of \textit{R249S} levels may provide a marker for early detection of HCC (50). Further studies are however needed to assess the temporal variations of \textit{R249S} levels in the plasma of subjects in the months and years that precede HCC diagnosis in order to identify trends that distinguish seasonally high levels in exposed subjects from pathologically high levels in subjects with precursor disease or early cancer.

\textbf{Conclusions and Perspectives}

Studies on \textit{TP53 R249S} mutations in relation with exposure to AFB1 and to risk of HCC have identified several unexpected features that shed new light on future approaches aimed at discovering the molecular signature of environmental carcinogens in liver cancer. First, and foremost, these studies have demonstrated the usefulness of DNA isolated from plasma or serum as surrogate material to detect mutations, not only in subjects with a diagnosed chronic liver disease but also in asymptomatic subjects exposed to a carcinogen, AFB1. This is however not the only instance where mutations are detectable in blood samples of healthy individuals. In heavy smokers, mutations known to be induced by tobacco carcinogens are detected in CFDNA even in the absence of any defined lung pathology (51). In a prospective study on cancer risk in a cohort of never-smokers, we have shown that mutations in \textit{TP53} and in \textit{KRA S} could be detected in CFDNA up to several years ahead of diagnosis of specific cancers including bladder cancer (52). These mutations were interpreted as the result of ongoing carcinogenic exposure rather than of the shedding of DNA by undiagnosed, sub-symptomatic lesions. Second, in the context of chronic and widespread exposure to an environmental carcinogen such as AFB1, mutations can form simultaneously in very large populations of cells, only a small minority of which will eventually proceed to cancer. Indeed, to sustain the observed copy numbers of \textit{R249S} DNA in the plasma of healthy chronic carriers, one should consider that the DNA of at least 50,000,000 cells with \textit{R249S} mutation must be shed every day in the plasma. Given that this DNA represents only a tiny fraction of daily cell turnover in the liver, it follows that \textit{R249S} mutations occur in hepatocytes at a rate much higher than previously considered. This rate is not compatible with the view that the mutation in \textit{TP53} may be an “initiating event” sufficient to launch cells on the path to cancer. Rather, the formation and accumulation of cells with \textit{R249S} may be the hallmark of an adaptive process during which cells under severe inflammatory stress acquire the capacity to escape massive liver destruction by mutating a critical gene involved in stress-induced apoptosis. This hypothesis entails that acquisition of \textit{R249S} mutations may provide liver cells with some form of protection against liver destruction, at the expense of maintaining cells with damaged genomes that are at greater risk of neoplastic evolution. In line with this hypothesis, it is interesting to note that severe liver cirrhosis preceding HCC diagnosis is less frequent in areas with AFB1 exposure and chronic HB carriage than in other areas of the world. In a nutshell, it is tempting to speculate that both risk factors somehow cooperate, not only in increasing cancer risk, but also in protecting against the most direct and prevalent consequence of chronic infection in an adverse environment, namely, chronic liver cirrhosis. Future studies are needed to identify the molecular signatures of other mutagenic processes in liver cancer and to understand how these mutations interfere with chronic infectious processes.

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