

This paper is dedicated to Professor David Shugar on the occasion of his 80th birthday

Tyrosine and its catabolites: from disease to cancer*

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Hereditary tyrosinemia type I (HT I, McKusick 276700) is a metabolic disease with a pattern of autosomal recessive inheritance. The disease is caused by a deficiency of the enzyme involved in the last step in the degradation of the amino acid tyrosine, fumarylacetoacetate hydrolase (FAH). The result of this block is the accumulation of catabolites some of which have been proposed to be highly toxic due to their alkylating potential. In humans, hereditary tyrosinemia is often associated with the development of hepatocellular carcinoma in young patients. The reasons for the high incidence of hepatocellular carcinoma are unknown but it has been suggested that it may be caused by accumulated metabolites such as fumarylacetoacetate (FAA) and maleylacetoacetate (MAA). The various mutational defects in the FAH gene are reviewed. The use of two mouse models of this disease to study the molecular basis of the pathologies associated with HT I are discussed. Finally, some preliminary data on the mutagenic potential of FAA and MAA in a gene reversal assay are presented.

HEREDITARY TYROSINEMIA: A SEVERE DISEASE OF TYROSINE CATABOLISM

Tyrosine is a semi-essential amino acid derived in mammals from the hydrolysis of dietary proteins and/or from the hydroxylation of the essential amino acid phenylalanine by phenylalanine hydroxylase (PAH) (review

in [1, 2]). As shown in Fig. 1, tyrosine can either be anabolysed for the production of catecholamines and melanins or degraded through a five-step catabolic pathway. The five enzymes involved in this degradative pathway are tyrosine aminotransferase (TAT), *p*-hydroxyphenylpyruvic acid dioxygenase (HPPD), homogentisic acid dioxygenase (HGAD), maleylacetoacetate isomerase (MAI) and fumarylacetoacetate hydrolase (FAH; EC 3.7.1.2). The

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Abbreviations: EMS, ethyl methanesulfonate; FAA, fumarylacetoacetate; FAH, fumarylacetoacetate hydrolase; HGAD, homogentisic acid dioxygenase; HGPRT, hypoxanthine-guanine phosphoryl transferase; HPPD, *p*-hydroxyphenylpyruvic acid dioxygenase; HT I, hereditary tyrosinemia type I; MAA, maleylacetoacetate; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; PAH, phenylalanine hydroxylase; SA, succinylacetone; TAT, tyrosine aminotransferase.

end products of the catabolic degradation are then incorporated into the citric acid cycle.

Inborn errors of metabolism have been documented for at least four of the steps of this pathway but the most severe disease, hereditary tyrosinemia type I (HT I), is the one affecting the last enzyme in the catabolic pathway, FAH. The enzymatic defect in HT I was originally described by Lindblad *et al.* [3] on the basis of the presence of succinylacetone (SA), a derivative of FAA, in urine of the patients. This compound which can be easily measured by its inhibitory activity on the enzyme δ -aminolevulinic acid dehydratase is diagnostic of the disease [3, 4].

Tyrosinemic children show as a rule hepatomegaly and have high levels of urinary and plasmatic tyrosine and methionine and of other metabolites of the catabolic pathway. Some of the clinical and biochemical features of HT I are summarized in Table 1. As can be seen, the clinical picture is variable with multiple pathological manifestations at the hepatic and renal levels. Another frequent feature of HT I is the occurrence of severe neurological porphyria-like crises [5]. There exist two clinical forms of tyrosinemia. The acute form of the disease is characterized by a rapid deterioration of he-

patic and renal functions leading to death from hepatic failure in infancy. In the chronic form, further complications arise with renal dysfunctions, cirrhosis and frequently development of hepatocellular carcinoma. HT I has been reported worldwide but shows a particularly high incidence in the French-Canadian population of eastern Quebec (Canada) where 1 in 1846 children are affected at birth [6].

In view of the high incidence of the disease in this region (Saguenay-Lac-St-Jean) where estimates of the frequency of carriers ranged from 1 in 16 to 1 in 25 [7, 8], and of its severity, efforts have been initiated a few years ago to find the molecular defect(s) involved in both clinical forms of HT I with the hope of devising simple tests for carriers' detection which could be applied to the population at risk.

The putative defective enzyme, FAH was purified from human, rat [9, 10] and beef liver [11] and used to generate antibodies to probe liver specimens from HT I patients and to clone the corresponding cDNA from expression libraries [12, 13]. By immunoblotting with an anti-rat FAH antibody, we showed that FAH was absent from liver extracts of HT I patients with the acute form of the disease and present, but at a reduced level correlated with the

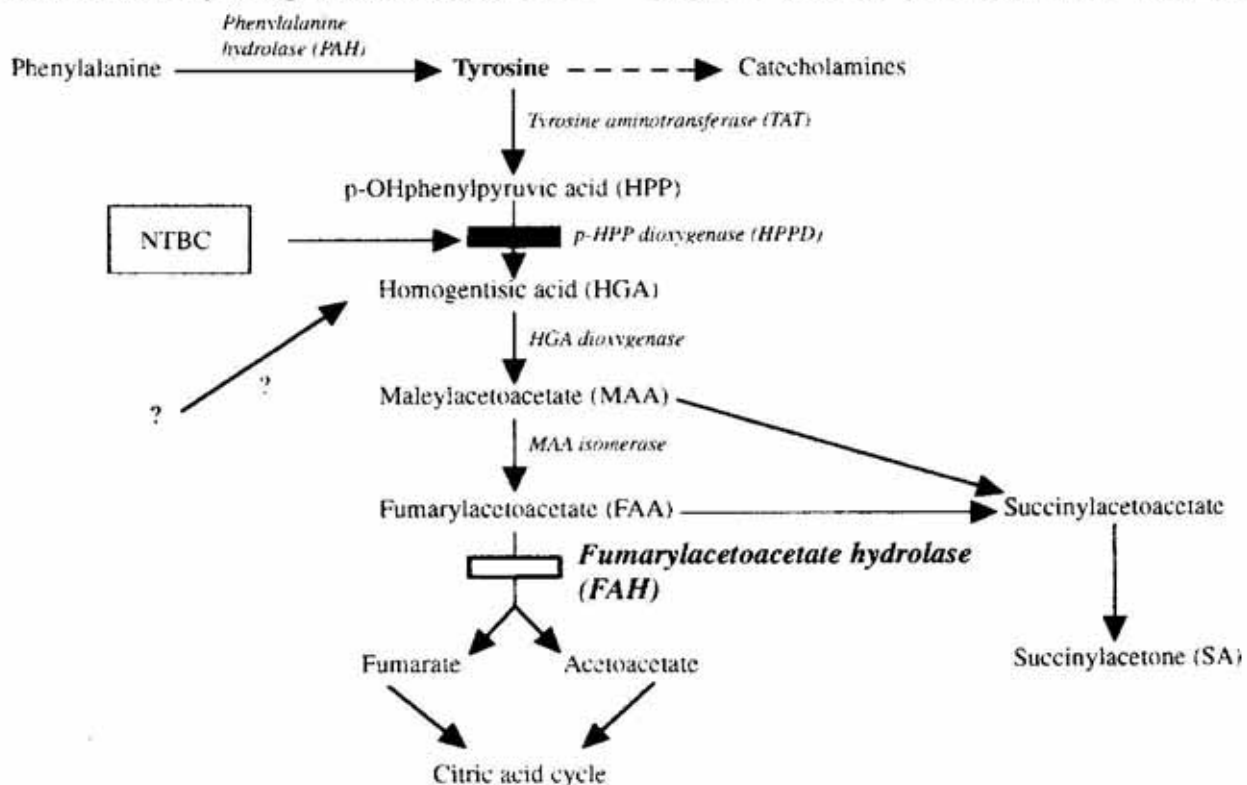


Fig. 1. Tyrosine catabolic pathway in mammals.

Table 1
Symptoms for hereditary tyrosinemia type I

SYMPTOMS	
Vomiting	Fever
Diarrhea	Lethargy
Failure to thrive	Irritability
SIGNS	
Hepatomegaly	Ascites
Jaundice	Bleeding tendencies
"Boiled cabbage" odor	
BIOCHEMISTRY	
Tyrosinemia	Tyrosyluria
Methioninemia	Glucosuria
Hyperbilirubinemia	Phosphaturia
Hypoglycemia	Fanconi-like syndrome with generalized aminoaciduria
Hypoprothrombinemia	
Hypoproteinemia	
Increased α -fetoprotein	
PATHOLOGY	
Rickets	Cirrhosis
Porphyria-like syndrome	Hepatic carcinoma
Neurologic crises	

measured enzymatic activity, in those from patients with the chronic form suggesting that the molecular basis of the defect was different in these two clinical forms [9, 14].

The human FAH gene which maps to region q23-q25 on human chromosome 15 [13] con-

tains 14 exons spanning over 35 kb of DNA [15]. The structure of the gene is identical to that of the mouse gene and the open reading frame shows 94% amino acids identity with the rat and mouse cDNAs (80% at the nucleotide level). FAH is mainly expressed in the liver and kidneys but also shows a basic level of expression in most tissues. A high level of expression of FAH has also been reported in specific cells of the white matter in the brain [16]. This is consistent with a preliminary analysis of the FAH gene promoter regions which is GC rich with no TATA nor CAAT elements. Eleven putative SP1 sites are localized in the first 600 base pairs [15]. Thus the FAH gene has elements common with housekeeping genes.

Probes and antibodies were used to investigate the molecular heterogeneity of HT I by northern blot, immunoblot and direct enzymatic activity measurements on a number of patients' liver specimens [14]. Some of the patients were chosen for mutational analysis by mRNA amplification and sequencing. The first discovered mutation of a French-Canadian patient was a missense mutation where an asparagine residue was substituted by isoleucine at position 16 (N16I). By means of site-directed mutagenesis of the FAH cDNA coupled with transfection in cultured cells and assay of the phenotype this mutation was shown to be the cause of HT I [14]. The transfected cells produced mRNA but no stable protein, a phenotype identical to that observed in the patient liver. This suggested that the N16I modification gave rise to an unstable enzyme. Unfortunately, this mutation turned out to be a rare one and

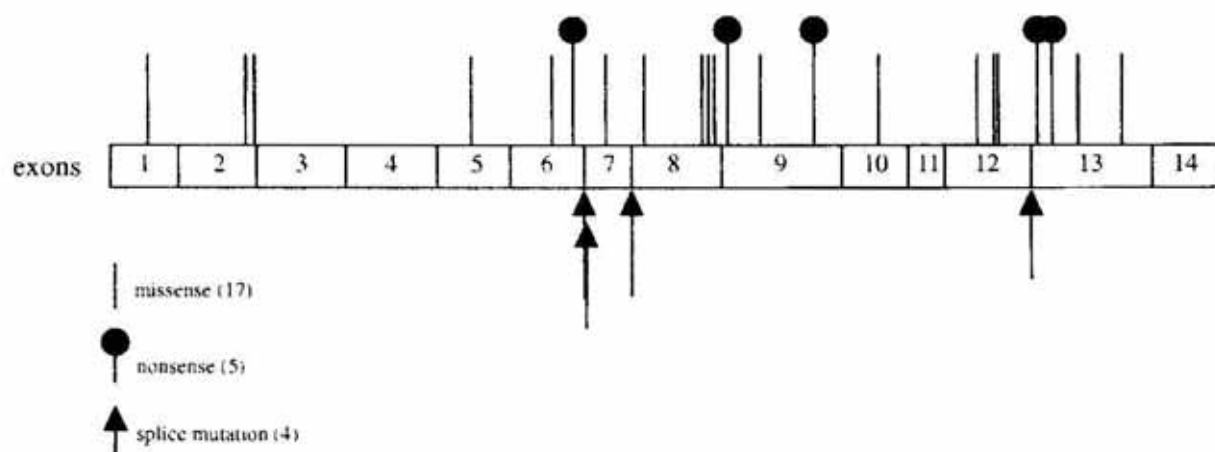


Fig. 2. Location of mutations found in the human FAH gene.

has only been found in one of the thirty-seven (37) HT I patients' DNA examined up to now.

In the past two years, many mutations have been reported in the FAH gene of HT I patients from various regions of the world and twenty seven (27) missense, splice or stop mutations have been reported at this time (M. St-Louis & R.M. Tanguay, submitted). As shown in Fig. 2, these mutations are evenly spread along the FAH gene with no evidence of concentration in any "hot spot" regions. Many of the mutations were found in single patients or within geographical isolates like the W262X mutation reported in the Finnish patients [17]. However, one mutation originally found in a French-Canadian and an Iranian HT I patients [18], the IVS12+5G→A splice mutation turned out to be the most prevalent and widely spread mutation of FAH. This mutation, which is prevalent in the eastern Québec French-Canadian population where it appears in up to 95.6% of alleles of carriers of HT I [8], has also been observed in 28% of HT I patients tested from outside Quebec [7]. This has led to the development of a carrier assay for this mutation within the population at risk as well as of other simple tests for frequent or geographically isolated mutations. We will now return to the issue of the aetiology of the pathologies in HT I.

As mentioned in the introduction, liver cancer is a common feature in a large percentage of HT I children [2]. The only effective treatment is liver transplantation and/or a drug treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a product which is presumed to act by preventing the accumulation of by-products resulting from the enzymatic block at the FAH level [19]. This inhibitor acts upstream of FAH by blocking HPPD, the second enzyme in the pathway (Fig. 1). Some of the by-products accumulating as a result of FAH deficiency are known to be highly toxic. This is the case for fumarylacetoacetate (FAA) and maleylacetoacetate (MAA) both of which have been suggested to be capable of alkylating thiols and other functional groups of biological macromolecules [1, 20].

Since there are yet no rigorous proofs that these products are causative of the active development of hepatocellular carcinoma in HT I patients, animal models would be particularly useful to study the basis of the pathology of this disease. Recently, two mouse models of HT I

have been described. The first one was obtained by transgenesis of a FAH cDNA into a well known neonatal lethal mouse model with a large deletion (4 Mb) on chromosome 7 [21]. This model known as the lethal albino mouse [22] has been extensively studied by many groups since many hepatic genes displayed abnormal expression or failed to respond to glucocorticoid induction at birth (review in [23]). Sequencing at the breakpoint and search of Genbank showed that the FAH gene was disrupted. Due to the large size of the deletion and to the complexity of the molecular phenotype of this mouse mutation, rescue experiments by transgenesis were initiated. The lethality phenotype could be corrected by simply expressing a FAH transgene [21]. Various mouse lines expressing different levels of the transgene were generated and are currently under study for assessing the effects of tyrosine catabolites on hepatic functions and on hepatocarcinogenesis. One of these lines (#921) seems particularly promising as it shows a low level of expression of FAH (< 4%) reminiscent of the phenotype in chronic human HT I patients. In this transgenic line rescue was not total and expression of the gene NMO-1 (encoding NAD(P)H — menadione oxidoreductase), a gene inducible by oxidative damage and involved in detoxification, remained high.

A second model was developed by Grompe and his colleagues who knocked-out the FAH gene by homologous recombination. The resulting mice died at birth thus reproducing the albino phenotype [24]. Although many hepatic functions were impaired in the homozygote recessive animal, the liver did not show the intensive damages seen in the liver of HT I patients. At first, this mouse model seemed of limited value since FAH^{-/-} homozygotes had a lethal phenotype. However, it was recently shown that pregnant mice treated with NTBC gave birth to normal fully viable homozygous FAH^{-/-} mice showing that the lethal hepatic dysfunction could be pharmacologically corrected [25]. Nevertheless, additional data on mice treated with NTBC shows that, on prolonged treatment 50% of them develop liver tumors [25].

Why does cancer develop in NTBC-treated mice? Three explanations are presently being examined. First, NTBC, a reversible inhibitor of HPPD [26], may incompletely block the path-

way at the dose used. A second possibility is that FAA is nevertheless produced by metabolites which can get in the degradation pathway by an unknown entry point downstream of HPPD. Such a pathway does exist in the fungus *Aspergillus nidulans* where phenylacetate can enter at the homogentisic acid step [27] (see Fig. 1). It remains to be seen if such alternative pathways exist in higher eukaryotes. A third possibility is that NTBC itself, which is a herbicide, is carcinogenic. However, at this time, the similarity in the liver pathology seen in HT I patients and in older mice on NTBC treatment suggests that the accumulation of, and exposure to, even small amounts of tyrosine by-products may have detrimental effects. Further studies will be needed to differentiate between these possibilities.

REVERSION OF MUTATIONS IN THE LIVERS OF HT I PATIENTS

Another recent finding of major interest is the reversion of the mutations in the liver of HT I

patients which was reported by Kvittingen *et al.* [28, 29]. Immunohistochemical studies showed a mosaic pattern of expression of FAH in the liver of five Norwegian patients. Amplification of DNA from the dissected FAH⁺ regions followed by sequencing showed reversion of the point mutation in one of the FAH allele. We also observed a similar reversion of FAH expression in the liver of French-Canadian patients homozygous for the splice mutation. Figure 3 shows a section from the liver of an HT I patient homozygous for the IVS12+5G→A splice mutation which was stained with an antibody against FAH. As can be seen, some nodules show expression of FAH. We have amplified FAH DNA from microdissected nodules and tested for the reversal of the mutation using a simple restriction enzyme assay. Reversion of the mutation was observed but the situation is not as simple as the one previously reported in the Norwegian group. Thus, reversion was observed in both FAH⁻ and FAH⁺ nodules. This intriguing preliminary observation is being further investigated to see whether this was the result of a staining artifact or whether other

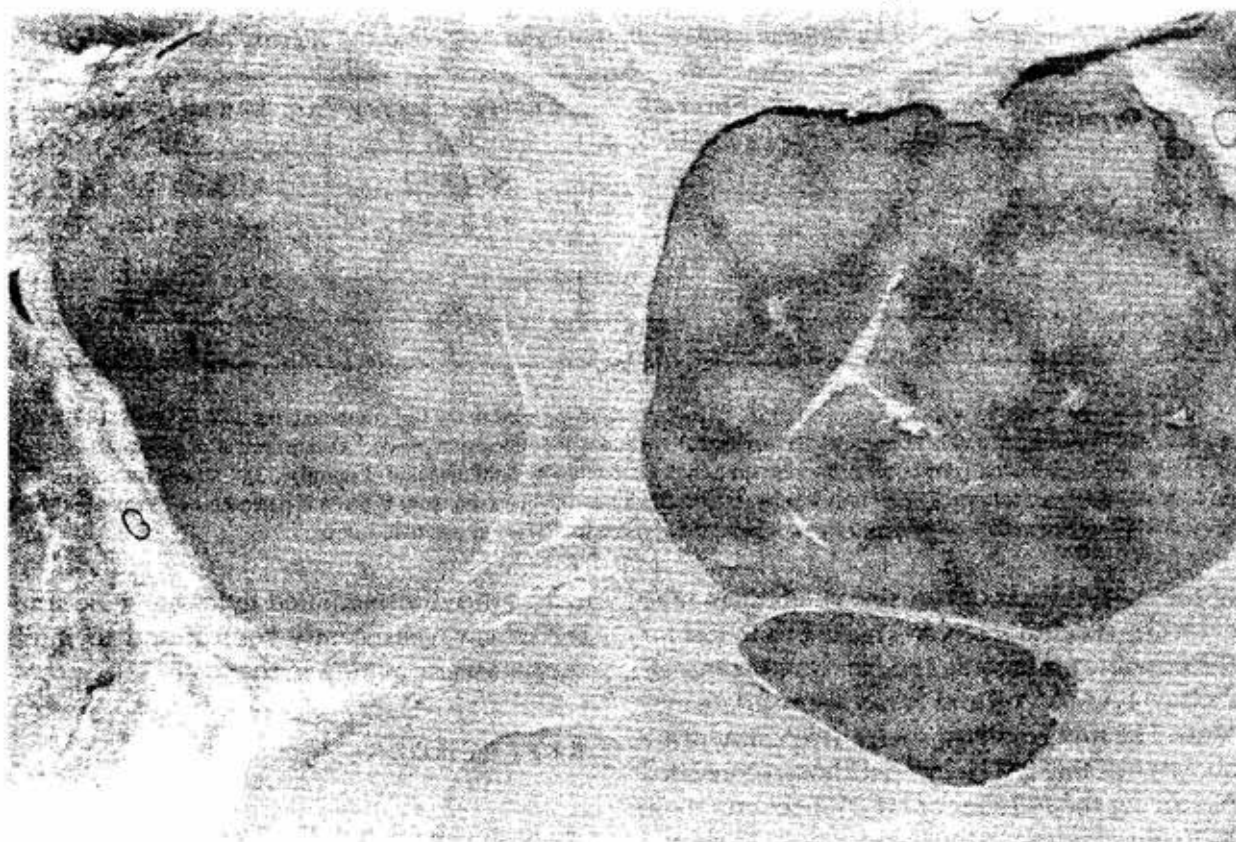


Fig. 3. FAH immunohistochemical staining by a standard peroxidase method on a 10 μ m liver section. This liver was obtained after transplantation of a 9 year old tyrosinemia patient homozygous for the IVS12+5G→A splice mutation. Two major ellipse-like nodules are seen. The one on the right has a large diameter of 4 mm and shows a strong level of FAH (FAH⁺). The one on the left has a large diameter of 3.5 mm and shows a low level of FAH (FAH⁻) (approx. 20 \times).

elements could control the expression of FAH in some nodules. In any event, these data suggest that FAH expressing cells have a selective growth advantage and that this property may be positively used to try to repopulate an hepatectomized liver transfected with, for example, an FAH containing retrovirus (see below).

ARE TYROSINE CATABOLITES CARCINOGENIC?

Previous tests conducted in our laboratory have shown that the reactive compounds FAA and MAA are toxic to cells and that this cytotoxic effect can be partially prevented by addition of glutathione (GSH) and other SH-containing molecules, a finding consistent with the adduction of FAA by glutathione (Tanguay, R.M. & Reed, A., unpublished). The discovery of mutation reversion in HT I livers, the extensive cellular damages observed in this organ and the high incidence of hepatocarcinomas (about 50%) in these patients suggest that some

these compounds is now being tested in different prokaryotic and eukaryotic assay systems.

GENE THERAPY IN HEREDITARY TYROSINEMIA

The successful rescue of the albino mouse which was observed even with a low level of FAH activity opens the possibility of using gene therapy as a means to complement the human enzyme deficient cells. Insertion of a human FAH cDNA in a retroviral vector has been shown to effectively restore FAH activity in primary fibroblasts from HT I patients [30]. Thus, it is therefore feasible in *ex-vivo* gene transfer experiments, such as that successfully used in the case of familial hypercholesterolemia [31], to restore FAH activity. Liver is an organ particularly appropriate for such an approach due to its regenerative potential and to the ease with which hepatocytes corrected *in vitro* can be re-targeted to the organ *in vivo*. It may also be possible to combine this approach

Table 2

Mutation frequency induced by fumarylacetoacetate, maleylacetoacetate and succinylacetone in V79 cells

Test compound	Concentration (μM)	Survival ^c (%)	Cloning efficiency (%)	Mutant frequency $\times 10^{-6}$ ^d
None ^a		100	48.2 \pm 7.2	6.8 \pm 0.8 (5.8–7.6) ^e
FAA ^b	100	48 \pm 15	69.2 \pm 7.5	16.4 \pm 4.4 (12.8–22.5)
MAA	100	84	83	7.2, 10.1 ^f
SA	700	90	88	7.7, 9.2
EMS ^g	2500	77	66	714.1, 485.5

^a Control cells were treated with the vehicle, HBSS. ^b The cells were exposed to the test compound for 24 h in DMEM/FBS (5%). ^c Determined from the cell number present at the first subculture during the expression period. ^d Determined after an expression period of 6 days, after which a total of 5×10^6 cells were subjected to selection by 6-thioguanine (7 $\mu\text{g}/\text{ml}$) [33]. ^e Values for untreated and FAA-treated cells are the mean \pm SE of four separate exposed cultures, the observed range is given in parentheses. ^f Values for MAA-, SA-, or EMS-treated cells were obtained from two single experiments. ^g Used as positive control.

of the by-products of tyrosine catabolism may even target cellular DNA. This led us to test for the possible mutagenic and/or carcinogenic effects of FAA, MAA and SA in appropriate systems. The first results of such an experiment are shown in Table 2. The system chosen consisted of scoring the number of HGPRT-revertants in V79 cells exposed to the compounds. As shown here, only FAA (100 μM) induced an increase in mutant frequency. The mutagenic potential of

with retroviral-mediated cytokine gene transfer which has recently been found to inhibit metastasis formation in the liver [32].

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